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<b>(21) International Application Number:</b> PCT/IB99/00520 <b>(22) International Filing Date:</b> 25 March 1999 (25.03.99) <b>(30) Priority Data:</b> 60/079,477 26 March 1998 (26.03.98) US <b>(71) Applicant (for all designated States except US):</b> THE PROCTER & GAMBLE COMPANY [US/US]; One Procter & Gamble Plaza, Cincinnati, OH 45202 (US). <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> RUBINGH, Donn, Nelton [US/US]; 8224 Sheed Road, Cincinnati, OH 45247 (US). SIKORSKI, Elizabeth, Ellen [US/US]; 5687 Genevieve Place, Fairfield, OH 45014 (US). CORREA, Paul, Elliott [US/US]; 4245 Miami Trails Lane, Cincinnati, OH 45252 (US). <b>(74) Agents:</b> REED, T., David et al.; The Procter & Gamble Company, 5299 Spring Grove Avenue, Cincinnati, OH 45217-1087 (US).		<b>(81) Designated States:</b> AL, AM, AT, AT (Utility model), AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, CZ (Utility model), DE, DE (Utility model), DK, DK (Utility model), EE, EE (Utility model), ES, FI, FI (Utility model), GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SK (Utility model), SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
<b>(54) Title:</b> SERINE PROTEASE VARIANTS HAVING AMINO ACID DELETIONS AND SUBSTITUTIONS		
<b>(57) Abstract</b> <p>The present invention relates to variants of serine proteases having decreased immunogenicity relative to their corresponding wild-type proteases. More particularly, the present invention relates to variants having a modified amino acid sequence of a wild-type amino acid sequence, wherein the modified amino acid sequence comprises a deletion and, optionally, a substitution of one or more specifically identified positions corresponding to subtilisin BPN'. The invention further relates to mutant genes encoding such variants and cleaning and personal care compositions comprising such variants.</p>		

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## SERINE PROTEASE VARIANTS HAVING AMINO ACID DELETIONS AND SUBSTITUTIONS

### FIELD OF THE INVENTION

The present invention relates to serine protease variants and compositions comprising the variants which have decreased immunogenicity relative to their corresponding wild-type serine proteases.

### BACKGROUND OF THE INVENTION

Enzymes make up the largest class of naturally occurring proteins. One class of enzyme includes proteases which catalyze the hydrolysis of other proteins. This ability to hydrolyze proteins has been exploited by incorporating naturally occurring and protein engineered proteases into cleaning compositions, particularly those relevant to laundry applications.

In the cleaning arts, the mostly widely utilized of these proteases are the serine proteases. Most of these serine proteases are produced by bacterial organisms while some are produced by other organisms, such as fungi. See Siezen, Roland J. et al., "Homology Modelling and Protein Engineering Strategy of Subtilases, the Family of Subtilisin-Like Serine Proteases", *Protein Engineering*, Vol. 4, No. 7, pp. 719 - 737 (1991). Unfortunately, the efficacy of the wild-type proteases in their natural environment frequently does not translate into the unnatural cleaning composition environment. Specifically, protease characteristics such as, for example, thermal stability, pH stability, oxidative stability and substrate specificity are not necessarily optimized for utilization outside the natural environment of the enzyme.

Several approaches have been employed to alter the wild-type amino acid sequence of serine proteases with the goal of increasing the efficacy of the protease in the unnatural wash environment. These approaches include the genetic redesign of proteases to enhance thermal stability and to improve oxidation stability under quite diverse conditions.

However, because such genetically engineered proteases are foreign to mammals, they are potential antigens. As antigens, these proteases cause immunological and

allergic responses (herein collectively described as immunological responses) in mammals. In fact, sensitization to serine proteases has been observed in environments wherein humans are regularly exposed to the proteases. Such environments include manufacturing facilities, where employees are exposed to the proteases through such vehicles as uncontrolled dust or aerosolization. Aerosolization can result by the introduction of the protease into the lung, which is the route of protease exposure which causes the most dangerous response. Protease sensitization can also occur in the marketplace, where consumers' repeated use of products containing proteases may cause an allergic reaction.

Furthermore, while genetic engineering has been prominent in the continuing search for more highly effective proteases for use in laundry applications, genetically engineered proteases have been minimally utilized in personal care compositions and light duty detergents. A primary reason for the absence of engineered proteases in products such as, for example, soaps, gels, body washes, and shampoos, is due to the aforementioned problem of human sensitization leading to undesirable immunological responses. It would therefore be highly advantageous to provide a personal care composition which provides the cleansing properties of engineered proteases with minimized provocation of immunological responses.

One approach toward alleviating the immunological activity of a protease is through the redesign of one or more epitopes of the protease. Epitopes are those amino acid regions of an antigen which evoke an immunological response through the binding of antibodies or the presentation of processed antigens to T cells *via* a major histocompatibility complex protein (MHC). Changes in the epitopes can affect their efficiency as an antigen. See Walsh, B.J. and M.E.H. Howden, "A Method for the Detection of IgE Binding Sequences of Allergens Based on a Modification of Epitope Mapping", *Journal of Immunological Methods*, Vol. 121, pp. 275 - 280 (1989).

The present inventors have discovered that those serine proteases commonly known as subtilisins, including subtilisin BPN', have a prominent epitope region at amino acid positions 70 - 84 corresponding to BPN'. The present inventors have herein genetically redesigned such subtilisins to alleviate the immunogenic properties attributed to this epitope region. In so doing, the present inventors have discovered subtilisins

which evoke a decreased immunological response yet maintain their activity as an efficient cleansing protease. Accordingly, the present proteases are suitable for use in several types of compositions including, but not limited to, laundry, dish, hard surface, skin care, hair care, beauty care, oral, and contact lens compositions.

### SUMMARY OF THE INVENTION

The present invention relates to variants of serine proteases having decreased immunogenicity relative to their corresponding wild-type proteases. More particularly, the present invention relates to variants having a modified amino acid sequence of a wild-type amino acid sequence, wherein the modified amino acid sequence comprises a deletion and, optionally, a substitution of one or more of positions 70 – 84 corresponding to subtilisin BPN'. The invention further relates to mutant genes encoding such variants and cleaning and personal care compositions comprising such variants.

### DETAILED DESCRIPTION OF THE INVENTION

The essential components of the present invention are herein described below. Also included are non-limiting descriptions of various optional and preferred components useful in embodiments of the present invention.

The present invention can comprise, consist of, or consist essentially of any of the required or optional components and / or limitations described herein.

All percentages and ratios are calculated by weight unless otherwise indicated. All percentages are calculated based on the total composition unless otherwise indicated.

All component or composition levels are in reference to the active level of that component or composition, and are exclusive of impurities, for example, residual solvents or by-products, that may be present in commercially available sources.

All documents referred to herein, including all patents, patent applications, and printed publications, are hereby incorporated by reference in their entirety.

As used herein, abbreviations will be used to describe amino acids. Table I provides a list of abbreviations used herein:

### Table I

<u>Amino Acid</u>	<u>Three-letter Abbreviation</u>	<u>One-letter Abbreviation</u>
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic Acid	Asp	D
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic Acid	Glu	E
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

#### Definitions

As used herein, the term "mutation" refers to alterations in gene sequences and amino acid sequences produced by those gene sequences. Mutations may be deletions, substitutions, or additions of amino acid residues to the wild-type protein sequence.

As used herein, the term "wild-type" refers to a protein, herein specifically a protease, produced by unmutated organisms.

As used herein, the term "variant" means a protein, herein specifically a protease, having an amino acid sequence which differs from that of the wild-type protease.

As referred to herein, while the variants of the present invention are not limited to those of subtilisin BPN', all amino acid numbering is with reference to the amino acid sequence for subtilisin BPN' which is represented by SEQ ID NO:1. The amino acid sequence for subtilisin BPN' is further described by Wells, J.A., E. Ferrari, D.J. Henner, D.A. Estell, and E.Y. Chen, Nucleic Acids Research, Vol. II, 7911 - 7925 (1983), incorporated herein by reference.

#### Variants of the Present Invention

The present inventors have discovered an epitope region in serine proteases which corresponds to positions 70 - 84 of subtilisin BPN'. The present inventors have further discovered that one or more amino acid deletions and / or substitutions in the amino acid sequence of a wild-type serine protease provides variants which evoke a decreased allergenic and / or immune response relative to the corresponding wild-type serine protease.

As used herein, a variant may be designated by referring to the deleted amino acid positions which characterize the variant. For example, a variant of a serine protease having deletions at positions 70, 75, 76, 77, 78, 79, 80, 81, and 82 may be designated as  $\Delta$  70, 75 - 82. Similarly, substitutions may be indicated by providing the wild-type amino acid residue, followed by the position number, followed by the substituted amino acid residue to be substituted. Wherein the substituted amino acid residue may be any natural amino acid, the symbol "\*" is provided. Multiple substitutions comprising a variant are separated by the symbol "+". To illustrate, a substitution of valine for glycine at position 70 is designated either Gly70Val or G70V. A variant comprising both deletions and substitutions is designated by combining the aforementioned designations. For example, an example of a variant having a substitution at both positions 70 and 72, as well as deletions at positions 75 - 82 is designated as  $\Delta$  75 - 82, G70V + V72A.

The variants of the present invention are variants of serine proteases. As used herein, the term "serine protease" means a protease which has at least 50%, and preferably 80%, amino acid sequence identity with the sequences for one or more of a subtilisin-like serine protease. A discussion relating to subtilisin-like serine proteases and their homologies may be found in Siezen et al., "Homology Modelling and Protein

Engineering Strategy of Subtilases, the Family of Subtilisin-Like Serine Proteases", *Protein Engineering*, Vol. 4, No. 7, pp. 719 - 737 (1991). Preferred serine proteases for mutation include subtilisin BPN', subtilisin Carlsberg, subtilisin DY, subtilisin 309, proteinase K, and thermitase. The most preferred serine protease for mutation is subtilisin BPN'.

The variants of the present invention are variants of serine proteases having a modified amino acid sequence of a wild-type amino acid sequence, wherein the modified amino acid sequence comprises a deletion of one or more of positions 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, and 84 (70 - 84) corresponding to BPN'. Unless comprising a personal care composition, the variants herein are not  $\Delta$  75 - 83 or  $\Delta$  71.

More preferably, the variants of the present invention comprise a deletion of one or more of positions 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, and 83 (73 - 83), even more preferably one or more of 75, 76, 77, 78, 79, 80, 81, and 82 (75 - 82).

Preferred variants of the present invention include  $\Delta$  70, 75 - 82;  $\Delta$  75 - 82;  $\Delta$  70, 78, 79;  $\Delta$  70;  $\Delta$  75;  $\Delta$  76;  $\Delta$  78;  $\Delta$  79;  $\Delta$  81; and  $\Delta$  82. The more preferred variants include  $\Delta$  70, 75 - 82;  $\Delta$  75 - 82;  $\Delta$  70, 78, 79;  $\Delta$  70;  $\Delta$  78; and  $\Delta$  79.

The variants of the present invention may optionally, in addition to the one or more deletions, further comprise a substitution of one or more of positions 70 - 84 corresponding to subtilisin BPN'. Of course, if a deletion has been made at any given position, a substitution cannot be made at that position.

Substitutions in the epitope region are made by replacing the wild-type amino acid residue with another natural amino acid residue such as one given in Table I.

Tables 2 - 10 below exemplify non-limiting variants of the present invention. With respect to these tables, in describing the specific mutations the wild-type amino acid residue is given first and the corresponding position number is given second. Table 2 delineates preferred single deletion variants. Tables 3 - 10 delineate preferred variants having one or more deletions and, optionally, one or more substitutions. To illustrate, the first example of Table 3 reads "LEU 82 GLY 83". This example is meant to exemplify three different variants: one having deletions at positions 82 and 83 ( $\Delta$  82, 83), one having a deletion at position 82 and a substitution at 83 ( $\Delta$  82, G83\*), and one having a substitution at position 82 and a deletion at position 83 ( $\Delta$  83, L82\*).



Table 2

## Single Deletion Variants

VAL 84  
GLY 83  
LEU 82  
VAL 81  
GLY 80  
ILE 79  
SER 78  
ASN 77  
ASN 76  
LEU 75  
ALA 74  
ALA 73  
VAL 72  
GLY 70

Table 3

## Double Mutation Variants

LEU 82 GLY 83  
VAL 81 GLY 83  
VAL 81 LEU 82  
GLY 80 GLY 83  
GLY 80 LEU 82  
GLY 80 VAL 81  
ILE 79 GLY 83  
ILE 79 LEU 82  
ILE 79 VAL 81  
ILE 79 GLY 80  
SER 78 GLY 83  
SER 78 LEU 82  
SER 78 VAL 81  
SER 78 GLY 80  
SER 78 ILE 79

ASN 77 GLY 83  
ASN 77 LEU 82  
ASN 77 VAL 81  
ASN 77 GLY 80  
ASN 77 ILE 79  
ASN 77 SER 78  
ASN 76 GLY 83  
ASN 76 LEU 82  
ASN 76 VAL 81  
ASN 76 GLY 80  
ASN 76 ILE 79  
ASN 76 SER 78  
ASN 76 ASN 77  
LEU 75 GLY 83  
LEU 75 LEU 82  
LEU 75 VAL 81  
LEU 75 GLY 80  
LEU 75 ILE 79  
LEU 75 SER 78  
LEU 75 ASN 77  
LEU 75 ASN 76  
ALA 74 GLY 83  
ALA 74 LEU 82  
ALA 74 VAL 81  
ALA 74 GLY 80  
ALA 74 ILE 79  
ALA 74 SER 78  
ALA 74 ASN 77  
ALA 74 ASN 76  
ALA 74 LEU 75  
ALA 73 GLY 83  
ALA 73 LEU 82  
ALA 73 VAL 81  
ALA 73 GLY 80  
ALA 73 ILE 79  
ALA 73 SER 78

ALA 73 ASN 77  
ALA 73 ASN 76  
ALA 73 LEU 75  
ALA 73 ALA 74

Table 4

Triple Mutation Variants

VAL 81 LEU 82 GLY 83  
GLY 80 LEU 82 GLY 83  
GLY 80 VAL 81 GLY 83  
GLY 80 VAL 81 LEU 82  
ILE 79 LEU 82 GLY 83  
ILE 79 VAL 81 GLY 83  
ILE 79 VAL 81 LEU 82  
ILE 79 GLY 80 GLY 83  
ILE 79 GLY 80 LEU 82  
ILE 79 GLY 80 VAL 81  
SER 78 LEU 82 GLY 83  
SER 78 VAL 81 GLY 83  
SER 78 VAL 81 LEU 82  
SER 78 GLY 80 GLY 83  
SER 78 GLY 80 LEU 82  
SER 78 GLY 80 VAL 81  
SER 78 ILE 79 GLY 83  
SER 78 ILE 79 LEU 82  
SER 78 ILE 79 VAL 81  
SER 78 ILE 79 GLY 80  
ASN 77 LEU 82 GLY 83  
ASN 77 VAL 81 GLY 83  
ASN 77 VAL 81 LEU 82  
ASN 77 GLY 80 GLY 83  
ASN 77 GLY 80 LEU 82  
ASN 77 GLY 80 VAL 81  
ASN 77 ILE 79 GLY 83  
ASN 77 ILE 79 LEU 82  
ASN 77 ILE 79 VAL 81

ASN 77 ILE 79 GLY 80  
ASN 77 SER 78 GLY 83  
ASN 77 SER 78 LEU 82  
ASN 77 SER 78 VAL 81  
ASN 77 SER 78 GLY 80  
ASN 77 SER 78 ILE 79  
ASN 76 LEU 82 GLY 83  
ASN 76 VAL 81 GLY 83  
ASN 76 VAL 81 LEU 82  
ASN 76 GLY 80 GLY 83  
ASN 76 GLY 80 LEU 82  
ASN 76 GLY 80 VAL 81  
ASN 76 ILE 79 GLY 83  
ASN 76 ILE 79 LEU 82  
ASN 76 ILE 79 VAL 81  
ASN 76 ILE 79 GLY 80  
ASN 76 SER 78 GLY 83  
ASN 76 SER 78 LEU 82  
ASN 76 SER 78 VAL 81  
ASN 76 SER 78 GLY 80  
ASN 76 SER 78 ILE 79  
ASN 76 ASN 77 GLY 83  
ASN 76 ASN 77 LEU 82  
ASN 76 ASN 77 VAL 81  
ASN 76 ASN 77 GLY 80  
ASN 76 ASN 77 ILE 79  
ASN 76 ASN 77 SER 78  
LEU 75 LEU 82 GLY 83  
LEU 75 VAL 81 GLY 83  
LEU 75 VAL 81 LEU 82  
LEU 75 GLY 80 GLY 83  
LEU 75 GLY 80 LEU 82  
LEU 75 GLY 80 VAL 81  
LEU 75 ILE 79 GLY 83  
LEU 75 ILE 79 LEU 82  
LEU 75 ILE 79 VAL 81

LEU 75 ILE 79 GLY 80  
LEU 75 SER 78 GLY 83  
LEU 75 SER 78 LEU 82  
LEU 75 SER 78 VAL 81  
LEU 75 SER 78 GLY 80  
LEU 75 SER 78 ILE 79  
LEU 75 ASN 77 GLY 83  
LEU 75 ASN 77 LEU 82  
LEU 75 ASN 77 VAL 81  
LEU 75 ASN 77 GLY 80  
LEU 75 ASN 77 ILE 79  
LEU 75 ASN 77 SER 78  
LEU 75 ASN 76 GLY 83  
LEU 75 ASN 76 LEU 82  
LEU 75 ASN 76 VAL 81  
LEU 75 ASN 76 GLY 80  
LEU 75 ASN 76 ILE 79  
LEU 75 ASN 76 SER 78  
LEU 75 ASN 76 ASN 77  
ALA 74 LEU 82 GLY 83  
ALA 74 VAL 81 GLY 83  
ALA 74 VAL 81 LEU 82  
ALA 74 GLY 80 GLY 83  
ALA 74 GLY 80 LEU 82  
ALA 74 GLY 80 VAL 81  
ALA 74 ILE 79 GLY 83  
ALA 74 ILE 79 LEU 82  
ALA 74 ILE 79 VAL 81  
ALA 74 ILE 79 GLY 80  
ALA 74 SER 78 GLY 83  
ALA 74 SER 78 LEU 82  
ALA 74 SER 78 VAL 81  
ALA 74 SER 78 GLY 80  
ALA 74 SER 78 ILE 79  
ALA 74 ASN 77 GLY 83  
ALA 74 ASN 77 LEU 82

ALA 74 ASN 77 VAL 81  
ALA 74 ASN 77 GLY 80  
ALA 74 ASN 77 ILE 79  
ALA 74 ASN 77 SER 78  
ALA 74 ASN 76 GLY 83  
ALA 74 ASN 76 LEU 82  
ALA 74 ASN 76 VAL 81  
ALA 74 ASN 76 GLY 80  
ALA 74 ASN 76 ILE 79  
ALA 74 ASN 76 SER 78  
ALA 74 ASN 76 ASN 77  
ALA 74 LEU 75 GLY 83  
ALA 74 LEU 75 LEU 82  
ALA 74 LEU 75 VAL 81  
ALA 74 LEU 75 GLY 80  
ALA 74 LEU 75 ILE 79  
ALA 74 LEU 75 SER 78  
ALA 74 LEU 75 ASN 77  
ALA 74 LEU 75 ASN 76  
ALA 73 LEU 82 GLY 83  
ALA 73 VAL 81 GLY 83  
ALA 73 VAL 81 LEU 82  
ALA 73 GLY 80 GLY 83  
ALA 73 GLY 80 LEU 82  
ALA 73 GLY 80 VAL 81  
ALA 73 ILE 79 GLY 83  
ALA 73 ILE 79 LEU 82  
ALA 73 ILE 79 VAL 81  
ALA 73 ILE 79 GLY 80  
ALA 73 SER 78 GLY 83  
ALA 73 SER 78 LEU 82  
ALA 73 SER 78 VAL 81  
ALA 73 SER 78 GLY 80  
ALA 73 SER 78 ILE 79  
ALA 73 ASN 77 GLY 83  
ALA 73 ASN 77 LEU 82

ALA 73 ASN 77 VAL 81  
ALA 73 ASN 77 GLY 80  
ALA 73 ASN 77 ILE 79  
ALA 73 ASN 77 SER 78  
ALA 73 ASN 76 GLY 83  
ALA 73 ASN 76 LEU 82  
ALA 73 ASN 76 VAL 81  
ALA 73 ASN 76 GLY 80  
ALA 73 ASN 76 ILE 79  
ALA 73 ASN 76 SER 78  
ALA 73 ASN 76 ASN 77  
ALA 73 LEU 75 GLY 83  
ALA 73 LEU 75 LEU 82  
ALA 73 LEU 75 VAL 81  
ALA 73 LEU 75 GLY 80  
ALA 73 LEU 75 ILE 79  
ALA 73 LEU 75 SER 78  
ALA 73 LEU 75 ASN 77  
ALA 73 LEU 75 ASN 76  
ALA 73 ALA 74 GLY 83  
ALA 73 ALA 74 LEU 82  
ALA 73 ALA 74 VAL 81  
ALA 73 ALA 74 GLY 80  
ALA 73 ALA 74 ILE 79  
ALA 73 ALA 74 SER 78  
ALA 73 ALA 74 ASN 77  
ALA 73 ALA 74 ASN 76  
ALA 73 ALA 74 LEU 75

Table 5

Quadruple Mutation Variants

GLY 80 VAL 81 LEU 82 GLY 83  
ILE 79 VAL 81 LEU 82 GLY 83  
ILE 79 GLY 80 LEU 82 GLY 83  
ILE 79 GLY 80 VAL 81 GLY 83

ILE 79 GLY 80 VAL 81 LEU 82  
SER 78 VAL 81 LEU 82 GLY 83  
SER 78 GLY 80 LEU 82 GLY 83  
SER 78 GLY 80 VAL 81 GLY 83  
SER 78 GLY 80 VAL 81 LEU 82  
SER 78 ILE 79 LEU 82 GLY 83  
SER 78 ILE 79 VAL 81 GLY 83  
SER 78 ILE 79 VAL 81 LEU 82  
SER 78 ILE 79 GLY 80 GLY 83  
SER 78 ILE 79 GLY 80 LEU 82  
SER 78 ILE 79 GLY 80 VAL 81  
ASN 77 VAL 81 LEU 82 GLY 83  
ASN 77 GLY 80 LEU 82 GLY 83  
ASN 77 GLY 80 VAL 81 GLY 83  
ASN 77 GLY 80 VAL 81 LEU 82  
ASN 77 ILE 79 LEU 82 GLY 83  
ASN 77 ILE 79 VAL 81 GLY 83  
ASN 77 ILE 79 VAL 81 LEU 82  
ASN 77 ILE 79 GLY 80 GLY 83  
ASN 77 ILE 79 GLY 80 LEU 82  
ASN 77 ILE 79 GLY 80 VAL 81  
ASN 77 SER 78 LEU 82 GLY 83  
ASN 77 SER 78 VAL 81 GLY 83  
ASN 77 SER 78 VAL 81 LEU 82  
ASN 77 SER 78 GLY 80 GLY 83  
ASN 77 SER 78 GLY 80 LEU 82  
ASN 77 SER 78 GLY 80 VAL 81  
ASN 77 SER 78 ILE 79 GLY 83  
ASN 77 SER 78 ILE 79 LEU 82  
ASN 77 SER 78 ILE 79 VAL 81  
ASN 77 SER 78 ILE 79 GLY 80  
ASN 76 VAL 81 LEU 82 GLY 83  
ASN 76 GLY 80 LEU 82 GLY 83  
ASN 76 GLY 80 VAL 81 GLY 83  
ASN 76 GLY 80 VAL 81 LEU 82  
ASN 76 ILE 79 LEU 82 GLY 83



ASN 76 ILE 79 VAL 81 GLY 83  
ASN 76 ILE 79 VAL 81 LEU 82  
ASN 76 ILE 79 GLY 80 GLY 83  
ASN 76 ILE 79 GLY 80 LEU 82  
ASN 76 ILE 79 GLY 80 VAL 81  
ASN 76 SER 78 LEU 82 GLY 83  
ASN 76 SER 78 VAL 81 GLY 83  
ASN 76 SER 78 VAL 81 LEU 82  
ASN 76 SER 78 GLY 80 GLY 83  
ASN 76 SER 78 GLY 80 LEU 82  
ASN 76 SER 78 GLY 80 VAL 81  
ASN 76 SER 78 ILE 79 GLY 83  
ASN 76 SER 78 ILE 79 LEU 82  
ASN 76 SER 78 ILE 79 VAL 81  
ASN 76 SER 78 ILE 79 GLY 80  
ASN 76 ASN 77 LEU 82 GLY 83  
ASN 76 ASN 77 VAL 81 GLY 83  
ASN 76 ASN 77 VAL 81 LEU 82  
ASN 76 ASN 77 GLY 80 GLY 83  
ASN 76 ASN 77 GLY 80 LEU 82  
ASN 76 ASN 77 GLY 80 VAL 81  
ASN 76 ASN 77 ILE 79 GLY 83  
ASN 76 ASN 77 ILE 79 LEU 82  
ASN 76 ASN 77 ILE 79 VAL 81  
ASN 76 ASN 77 ILE 79 GLY 80  
ASN 76 ASN 77 SER 78 GLY 83  
ASN 76 ASN 77 SER 78 LEU 82  
ASN 76 ASN 77 SER 78 VAL 81  
ASN 76 ASN 77 SER 78 GLY 80  
ASN 76 ASN 77 SER 78 ILE 79  
LEU 75 VAL 81 LEU 82 GLY 83  
LEU 75 GLY 80 LEU 82 GLY 83  
LEU 75 GLY 80 VAL 81 GLY 83  
LEU 75 GLY 80 VAL 81 LEU 82  
LEU 75 ILE 79 LEU 82 GLY 83  
LEU 75 ILE 79 VAL 81 GLY 83

LEU 75 ILE 79 VAL 81 LEU 82  
LEU 75 ILE 79 GLY 80 GLY 83  
LEU 75 ILE 79 GLY 80 LEU 82  
LEU 75 ILE 79 GLY 80 VAL 81  
LEU 75 SER 78 LEU 82 GLY 83  
LEU 75 SER 78 VAL 81 GLY 83  
LEU 75 SER 78 VAL 81 LEU 82  
LEU 75 SER 78 GLY 80 GLY 83  
LEU 75 SER 78 GLY 80 LEU 82  
LEU 75 SER 78 GLY 80 VAL 81  
LEU 75 SER 78 ILE 79 GLY 83  
LEU 75 SER 78 ILE 79 LEU 82  
LEU 75 SER 78 ILE 79 VAL 81  
LEU 75 SER 78 ILE 79 GLY 80  
LEU 75 ASN 77 LEU 82 GLY 83  
LEU 75 ASN 77 VAL 81 GLY 83  
LEU 75 ASN 77 VAL 81 LEU 82  
LEU 75 ASN 77 GLY 80 GLY 83  
LEU 75 ASN 77 GLY 80 LEU 82  
LEU 75 ASN 77 GLY 80 VAL 81  
LEU 75 ASN 77 ILE 79 GLY 83  
LEU 75 ASN 77 ILE 79 LEU 82  
LEU 75 ASN 77 ILE 79 VAL 81  
LEU 75 ASN 77 ILE 79 GLY 80  
LEU 75 ASN 77 SER 78 GLY 83  
LEU 75 ASN 77 SER 78 LEU 82  
LEU 75 ASN 77 SER 78 VAL 81  
LEU 75 ASN 77 SER 78 GLY 80  
LEU 75 ASN 77 SER 78 ILE 79  
LEU 75 ASN 76 LEU 82 GLY 83  
LEU 75 ASN 76 VAL 81 GLY 83  
LEU 75 ASN 76 VAL 81 LEU 82  
LEU 75 ASN 76 GLY 80 GLY 83  
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LEU 75 ASN 76 GLY 80 VAL 81  
LEU 75 ASN 76 ILE 79 GLY 83

LEU 75 ASN 76 ILE 79 LEU 82  
LEU 75 ASN 76 ILE 79 VAL 81  
LEU 75 ASN 76 ILE 79 GLY 80  
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LEU 75 ASN 76 SER 78 LEU 82  
LEU 75 ASN 76 SER 78 VAL 81  
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LEU 75 ASN 76 SER 78 ILE 79  
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LEU 75 ASN 76 ASN 77 LEU 82  
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ALA 74 ILE 79 GLY 80 VAL 81  
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ALA 73 ALA 74 LEU 75 LEU 82  
ALA 73 ALA 74 LEU 75 VAL 81  
ALA 73 ALA 74 LEU 75 GLY 80  
ALA 73 ALA 74 LEU 75 ILE 79  
ALA 73 ALA 74 LEU 75 SER 78



ALA 73 ALA 74 LEU 75 ASN 77

ALA 73 ALA 74 LEU 75 ASN 76

Table 6

## Quintuple Mutation Variants

ILE 79 GLY 80 VAL 81 LEU 82 GLY 83  
SER 78 GLY 80 VAL 81 LEU 82 GLY 83  
SER 78 ILE 79 VAL 81 LEU 82 GLY 83  
SER 78 ILE 79 GLY 80 LEU 82 GLY 83  
SER 78 ILE 79 GLY 80 VAL 81 GLY 83  
SER 78 ILE 79 GLY 80 VAL 81 LEU 82  
ASN 77 GLY 80 VAL 81 LEU 82 GLY 83  
ASN 77 ILE 79 VAL 81 LEU 82 GLY 83  
ASN 77 ILE 79 GLY 80 LEU 82 GLY 83  
ASN 77 ILE 79 GLY 80 VAL 81 GLY 83  
ASN 77 ILE 79 GLY 80 VAL 81 LEU 82  
ASN 77 SER 78 VAL 81 LEU 82 GLY 83  
ASN 77 SER 78 GLY 80 LEU 82 GLY 83  
ASN 77 SER 78 GLY 80 VAL 81 GLY 83  
ASN 77 SER 78 GLY 80 VAL 81 LEU 82  
ASN 77 SER 78 ILE 79 LEU 82 GLY 83  
ASN 77 SER 78 ILE 79 VAL 81 GLY 83  
ASN 77 SER 78 ILE 79 VAL 81 LEU 82  
ASN 77 SER 78 ILE 79 GLY 80 GLY 83  
ASN 77 SER 78 ILE 79 GLY 80 LEU 82  
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ASN 76 ILE 79 GLY 80 VAL 81 GLY 83  
ASN 76 ILE 79 GLY 80 VAL 81 LEU 82  
ASN 76 SER 78 VAL 81 LEU 82 GLY 83  
ASN 76 SER 78 GLY 80 LEU 82 GLY 83  
ASN 76 SER 78 GLY 80 VAL 81 GLY 83  
ASN 76 SER 78 GLY 80 VAL 81 LEU 82  
ASN 76 SER 78 ILE 79 LEU 82 GLY 83

ASN 76 SER 78 ILE 79 VAL 81 GLY 83  
ASN 76 SER 78 ILE 79 VAL 81 LEU 82  
ASN 76 SER 78 ILE 79 GLY 80 GLY 83  
ASN 76 SER 78 ILE 79 GLY 80 LEU 82  
ASN 76 SER 78 ILE 79 GLY 80 VAL 81  
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LEU 75 SER 78 VAL 81 LEU 82 GLY 83  
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LEU 75 ASN 76 SER 78 VAL 81 GLY 83

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ALA 73 ASN 77 SER 78 GLY 80 LEU 82  
ALA 73 ASN 77 SER 78 GLY 80 VAL 81



ALA 73 ASN 77 SER 78 ILE 79 GLY 83  
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ALA 73 LEU 75 SER 78 ILE 79 GLY 80  
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ALA 73 ALA 74 LEU 75 ASN 76 ILE 79  
ALA 73 ALA 74 LEU 75 ASN 76 SER 78  
ALA 73 ALA 74 LEU 75 ASN 76 ASN 77

Table 7

## Sextuple Mutation Variants

SER 78 ILE 79 GLY 80 VAL 81 LEU 82 GLY 83  
ASN 77 ILE 79 GLY 80 VAL 81 LEU 82 GLY 83  
ASN 77 SER 78 GLY 80 VAL 81 LEU 82 GLY 83  
ASN 77 SER 78 ILE 79 VAL 81 LEU 82 GLY 83  
ASN 77 SER 78 ILE 79 GLY 80 LEU 82 GLY 83  
ASN 77 SER 78 ILE 79 GLY 80 VAL 81 GLY 83  
ASN 77 SER 78 ILE 79 GLY 80 VAL 81 LEU 82  
ASN 76 ILE 79 GLY 80 VAL 81 LEU 82 GLY 83  
ASN 76 SER 78 GLY 80 VAL 81 LEU 82 GLY 83  
ASN 76 SER 78 ILE 79 VAL 81 LEU 82 GLY 83  
ASN 76 SER 78 ILE 79 GLY 80 LEU 82 GLY 83  
ASN 76 SER 78 ILE 79 GLY 80 VAL 81 GLY 83  
ASN 76 SER 78 ILE 79 GLY 80 VAL 81 LEU 82  
ASN 76 ASN 77 GLY 80 VAL 81 LEU 82 GLY 83  
ASN 76 ASN 77 ILE 79 VAL 81 LEU 82 GLY 83  
ASN 76 ASN 77 ILE 79 GLY 80 LEU 82 GLY 83  
ASN 76 ASN 77 ILE 79 GLY 80 VAL 81 GLY 83  
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ASN 76 ASN 77 SER 78 GLY 80 LEU 82 GLY 83  
ASN 76 ASN 77 SER 78 GLY 80 VAL 81 GLY 83  
ASN 76 ASN 77 SER 78 GLY 80 VAL 81 LEU 82  
ASN 76 ASN 77 SER 78 ILE 79 LEU 82 GLY 83  
ASN 76 ASN 77 SER 78 ILE 79 VAL 81 GLY 83  
ASN 76 ASN 77 SER 78 ILE 79 VAL 81 LEU 82  
ASN 76 ASN 77 SER 78 ILE 79 GLY 80 GLY 83  
ASN 76 ASN 77 SER 78 ILE 79 GLY 80 LEU 82  
ASN 76 ASN 77 SER 78 ILE 79 GLY 80 VAL 81  
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LEU 75 SER 78 GLY 80 VAL 81 LEU 82 GLY 83  
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LEU 75 SER 78 ILE 79 GLY 80 LEU 82 GLY 83  
LEU 75 SER 78 ILE 79 GLY 80 VAL 81 GLY 83  
LEU 75 SER 78 ILE 79 GLY 80 VAL 81 LEU 82

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 ALA 73 ALA 74 LEU 75 ASN 76 ASN 77 ILE 79  
 ALA 73 ALA 74 LEU 75 ASN 76 ASN 77 SER 78

Table 8

## Septuple Mutation Variants

ASN 77 SER 78 ILE 79 GLY 80 VAL 81 LEU 82 GLY 83

ASN 76 SER 78 ILE 79 GLY 80 VAL 81 LEU 82 GLY 83  
ASN 76 ASN 77 ILE 79 GLY 80 VAL 81 LEU 82 GLY 83  
ASN 76 ASN 77 SER 78 GLY 80 VAL 81 LEU 82 GLY 83  
ASN 76 ASN 77 SER 78 ILE 79 VAL 81 LEU 82 GLY 83  
ASN 76 ASN 77 SER 78 ILE 79 GLY 80 LEU 82 GLY 83  
ASN 76 ASN 77 SER 78 ILE 79 GLY 80 VAL 81 GLY 83  
ASN 76 ASN 77 SER 78 ILE 79 GLY 80 VAL 81 LEU 82  
LEU 75 SER 78 ILE 79 GLY 80 VAL 81 LEU 82 GLY 83  
LEU 75 ASN 77 ILE 79 GLY 80 VAL 81 LEU 82 GLY 83  
LEU 75 ASN 77 SER 78 GLY 80 VAL 81 LEU 82 GLY 83  
LEU 75 ASN 77 SER 78 ILE 79 VAL 81 LEU 82 GLY 83  
LEU 75 ASN 77 SER 78 ILE 79 GLY 80 LEU 82 GLY 83  
LEU 75 ASN 77 SER 78 ILE 79 GLY 80 VAL 81 GLY 83  
LEU 75 ASN 77 SER 78 ILE 79 GLY 80 VAL 81 LEU 82  
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ALA 73 LEU 75 ASN 76 SER 78 GLY 80 LEU 82 GLY 83  
ALA 73 LEU 75 ASN 76 SER 78 GLY 80 VAL 81 GLY 83  
ALA 73 LEU 75 ASN 76 SER 78 GLY 80 VAL 81 LEU 82  
ALA 73 LEU 75 ASN 76 SER 78 ILE 79 LEU 82 GLY 83  
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ALA 73 LEU 75 ASN 76 SER 78 ILE 79 VAL 81 LEU 82

ALA 73 LEU 75 ASN 76 SER 78 ILE 79 GLY 80 GLY 83  
ALA 73 LEU 75 ASN 76 SER 78 ILE 79 GLY 80 LEU 82  
ALA 73 LEU 75 ASN 76 SER 78 ILE 79 GLY 80 VAL 81  
ALA 73 LEU 75 ASN 76 ASN 77 VAL 81 LEU 82 GLY 83  
ALA 73 LEU 75 ASN 76 ASN 77 GLY 80 LEU 82 GLY 83  
ALA 73 LEU 75 ASN 76 ASN 77 GLY 80 VAL 81 GLY 83  
ALA 73 LEU 75 ASN 76 ASN 77 GLY 80 VAL 81 LEU 82  
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ALA 73 LEU 75 ASN 76 ASN 77 ILE 79 VAL 81 GLY 83  
ALA 73 LEU 75 ASN 76 ASN 77 ILE 79 VAL 81 LEU 82  
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ALA 73 LEU 75 ASN 76 ASN 77 ILE 79 GLY 80 VAL 81  
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ALA 73 ALA 74 ASN 77 SER 78 GLY 80 LEU 82 GLY 83



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ALA 73 ALA 74 ASN 76 ASN 77 SER 78 VAL 81 LEU 82

ALA 73 ALA 74 ASN 76 ASN 77 SER 78 GLY 80 GLY 83  
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ALA 73 ALA 74 ASN 76 ASN 77 SER 78 ILE 79 GLY 80  
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ALA 73 ALA 74 LEU 75 ILE 79 GLY 80 VAL 81 LEU 82  
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ALA 73 ALA 74 LEU 75 SER 78 GLY 80 LEU 82 GLY 83  
ALA 73 ALA 74 LEU 75 SER 78 GLY 80 VAL 81 GLY 83  
ALA 73 ALA 74 LEU 75 SER 78 GLY 80 VAL 81 LEU 82  
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ALA 73 ALA 74 LEU 75 SER 78 ILE 79 VAL 81 GLY 83  
ALA 73 ALA 74 LEU 75 SER 78 ILE 79 VAL 81 LEU 82  
ALA 73 ALA 74 LEU 75 SER 78 ILE 79 GLY 80 GLY 83  
ALA 73 ALA 74 LEU 75 SER 78 ILE 79 GLY 80 LEU 82  
ALA 73 ALA 74 LEU 75 SER 78 ILE 79 GLY 80 VAL 81  
ALA 73 ALA 74 LEU 75 ASN 77 VAL 81 LEU 82 GLY 83  
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ALA 73 ALA 74 LEU 75 ASN 77 ILE 79 VAL 81 LEU 82  
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ALA 73 ALA 74 LEU 75 ASN 77 SER 78 LEU 82 GLY 83  
ALA 73 ALA 74 LEU 75 ASN 77 SER 78 VAL 81 GLY 83  
ALA 73 ALA 74 LEU 75 ASN 77 SER 78 VAL 81 LEU 82  
ALA 73 ALA 74 LEU 75 ASN 77 SER 78 GLY 80 GLY 83

ALA 73 ALA 74 LEU 75 ASN 77 SER 78 GLY 80 LEU 82  
ALA 73 ALA 74 LEU 75 ASN 77 SER 78 GLY 80 VAL 81  
ALA 73 ALA 74 LEU 75 ASN 77 SER 78 ILE 79 GLY 83  
ALA 73 ALA 74 LEU 75 ASN 77 SER 78 ILE 79 LEU 82  
ALA 73 ALA 74 LEU 75 ASN 77 SER 78 ILE 79 VAL 81  
ALA 73 ALA 74 LEU 75 ASN 77 SER 78 ILE 79 GLY 80  
ALA 73 ALA 74 LEU 75 ASN 76 VAL 81 LEU 82 GLY 83  
ALA 73 ALA 74 LEU 75 ASN 76 GLY 80 LEU 82 GLY 83  
ALA 73 ALA 74 LEU 75 ASN 76 GLY 80 VAL 81 GLY 83  
ALA 73 ALA 74 LEU 75 ASN 76 GLY 80 VAL 81 LEU 82  
ALA 73 ALA 74 LEU 75 ASN 76 ILE 79 LEU 82 GLY 83  
ALA 73 ALA 74 LEU 75 ASN 76 ILE 79 VAL 81 GLY 83  
ALA 73 ALA 74 LEU 75 ASN 76 ILE 79 VAL 81 LEU 82  
ALA 73 ALA 74 LEU 75 ASN 76 ILE 79 GLY 80 GLY 83  
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ALA 73 ALA 74 LEU 75 ASN 76 ILE 79 GLY 80 VAL 81  
ALA 73 ALA 74 LEU 75 ASN 76 SER 78 LEU 82 GLY 83  
ALA 73 ALA 74 LEU 75 ASN 76 SER 78 VAL 81 GLY 83  
ALA 73 ALA 74 LEU 75 ASN 76 SER 78 VAL 81 LEU 82  
ALA 73 ALA 74 LEU 75 ASN 76 SER 78 GLY 80 GLY 83  
ALA 73 ALA 74 LEU 75 ASN 76 SER 78 GLY 80 LEU 82  
ALA 73 ALA 74 LEU 75 ASN 76 SER 78 GLY 80 VAL 81  
ALA 73 ALA 74 LEU 75 ASN 76 SER 78 ILE 79 GLY 83  
ALA 73 ALA 74 LEU 75 ASN 76 SER 78 ILE 79 LEU 82  
ALA 73 ALA 74 LEU 75 ASN 76 SER 78 ILE 79 VAL 81  
ALA 73 ALA 74 LEU 75 ASN 76 SER 78 ILE 79 GLY 80  
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ALA 73 ALA 74 LEU 75 ASN 76 ASN 77 VAL 81 GLY 83  
ALA 73 ALA 74 LEU 75 ASN 76 ASN 77 VAL 81 LEU 82  
ALA 73 ALA 74 LEU 75 ASN 76 ASN 77 GLY 80 GLY 83  
ALA 73 ALA 74 LEU 75 ASN 76 ASN 77 GLY 80 LEU 82  
ALA 73 ALA 74 LEU 75 ASN 76 ASN 77 GLY 80 VAL 81  
ALA 73 ALA 74 LEU 75 ASN 76 ASN 77 ILE 79 GLY 83  
ALA 73 ALA 74 LEU 75 ASN 76 ASN 77 ILE 79 LEU 82  
ALA 73 ALA 74 LEU 75 ASN 76 ASN 77 ILE 79 VAL 81  
ALA 73 ALA 74 LEU 75 ASN 76 ASN 77 ILE 79 GLY 80

ALA 73 ALA 74 LEU 75 ASN 76 ASN 77 SER 78 GLY 83  
 ALA 73 ALA 74 LEU 75 ASN 76 ASN 77 SER 78 LEU 82  
 ALA 73 ALA 74 LEU 75 ASN 76 ASN 77 SER 78 VAL 81  
 ALA 73 ALA 74 LEU 75 ASN 76 ASN 77 SER 78 GLY 80  
 ALA 73 ALA 74 LEU 75 ASN 76 ASN 77 SER 78 ILE 79

Table 9

## Octuple Mutation Variants

ASN 76 ASN 77 SER 78 ILE 79 GLY 80 VAL 81 LEU 82 GLY 83  
 LEU 75 ASN 77 SER 78 ILE 79 GLY 80 VAL 81 LEU 82 GLY 83  
 LEU 75 ASN 76 SER 78 ILE 79 GLY 80 VAL 81 LEU 82 GLY 83  
 LEU 75 ASN 76 ASN 77 ILE 79 GLY 80 VAL 81 LEU 82 GLY 83  
 LEU 75 ASN 76 ASN 77 SER 78 GLY 80 VAL 81 LEU 82 GLY 83  
 LEU 75 ASN 76 ASN 77 SER 78 ILE 79 VAL 81 LEU 82 GLY 83  
 LEU 75 ASN 76 ASN 77 SER 78 ILE 79 GLY 80 LEU 82 GLY 83  
 LEU 75 ASN 76 ASN 77 SER 78 ILE 79 GLY 80 VAL 81 GLY 83  
 LEU 75 ASN 76 ASN 77 SER 78 ILE 79 GLY 80 VAL 81 LEU 82  
 ALA 74 ASN 77 SER 78 ILE 79 GLY 80 VAL 81 LEU 82 GLY 83  
 ALA 74 ASN 76 SER 78 ILE 79 GLY 80 VAL 81 LEU 82 GLY 83  
 ALA 74 ASN 76 ASN 77 ILE 79 GLY 80 VAL 81 LEU 82 GLY 83  
 ALA 74 ASN 76 ASN 77 SER 78 GLY 80 VAL 81 LEU 82 GLY 83  
 ALA 74 ASN 76 ASN 77 SER 78 ILE 79 VAL 81 LEU 82 GLY 83  
 ALA 74 ASN 76 ASN 77 SER 78 ILE 79 GLY 80 LEU 82 GLY 83  
 ALA 74 ASN 76 ASN 77 SER 78 ILE 79 GLY 80 VAL 81 GLY 83  
 ALA 74 ASN 76 ASN 77 SER 78 ILE 79 GLY 80 VAL 81 LEU 82  
 ALA 74 LEU 75 SER 78 ILE 79 GLY 80 VAL 81 LEU 82 GLY 83  
 ALA 74 LEU 75 ASN 77 ILE 79 GLY 80 VAL 81 LEU 82 GLY 83  
 ALA 74 LEU 75 ASN 77 SER 78 GLY 80 VAL 81 LEU 82 GLY 83  
 ALA 74 LEU 75 ASN 77 SER 78 ILE 79 VAL 81 LEU 82 GLY 83  
 ALA 74 LEU 75 ASN 77 SER 78 ILE 79 GLY 80 LEU 82 GLY 83  
 ALA 74 LEU 75 ASN 77 SER 78 ILE 79 GLY 80 VAL 81 GLY 83  
 ALA 74 LEU 75 ASN 77 SER 78 ILE 79 GLY 80 VAL 81 LEU 82  
 ALA 74 LEU 75 ASN 76 ILE 79 GLY 80 VAL 81 LEU 82 GLY 83  
 ALA 74 LEU 75 ASN 76 SER 78 GLY 80 VAL 81 LEU 82 GLY 83  
 ALA 74 LEU 75 ASN 76 SER 78 ILE 79 VAL 81 LEU 82 GLY 83  
 ALA 74 LEU 75 ASN 76 SER 78 ILE 79 GLY 80 LEU 82 GLY 83

ALA 74 LEU 75 ASN 76 SER 78 ILE 79 GLY 80 VAL 81 GLY 83  
ALA 74 LEU 75 ASN 76 SER 78 ILE 79 GLY 80 VAL 81 LEU 82  
ALA 74 LEU 75 ASN 76 ASN 77 GLY 80 VAL 81 LEU 82 GLY 83  
ALA 74 LEU 75 ASN 76 ASN 77 ILE 79 VAL 81 LEU 82 GLY 83  
ALA 74 LEU 75 ASN 76 ASN 77 ILE 79 GLY 80 LEU 82 GLY 83  
ALA 74 LEU 75 ASN 76 ASN 77 ILE 79 GLY 80 VAL 81 GLY 83  
ALA 74 LEU 75 ASN 76 ASN 77 ILE 79 GLY 80 VAL 81 LEU 82  
ALA 74 LEU 75 ASN 76 ASN 77 SER 78 VAL 81 LEU 82 GLY 83  
ALA 74 LEU 75 ASN 76 ASN 77 SER 78 GLY 80 LEU 82 GLY 83  
ALA 74 LEU 75 ASN 76 ASN 77 SER 78 GLY 80 VAL 81 GLY 83  
ALA 74 LEU 75 ASN 76 ASN 77 SER 78 GLY 80 VAL 81 LEU 82  
ALA 74 LEU 75 ASN 76 ASN 77 SER 78 ILE 79 LEU 82 GLY 83  
ALA 74 LEU 75 ASN 76 ASN 77 SER 78 ILE 79 VAL 81 GLY 83  
ALA 74 LEU 75 ASN 76 ASN 77 SER 78 ILE 79 VAL 81 LEU 82  
ALA 74 LEU 75 ASN 76 ASN 77 SER 78 ILE 79 GLY 80 GLY 83  
ALA 74 LEU 75 ASN 76 ASN 77 SER 78 ILE 79 GLY 80 LEU 82  
ALA 74 LEU 75 ASN 76 ASN 77 SER 78 ILE 79 GLY 80 VAL 81  
ALA 73 ASN 77 SER 78 ILE 79 GLY 80 VAL 81 LEU 82 GLY 83  
ALA 73 ASN 76 SER 78 ILE 79 GLY 80 VAL 81 LEU 82 GLY 83  
ALA 73 ASN 76 ASN 77 ILE 79 GLY 80 VAL 81 LEU 82 GLY 83  
ALA 73 ASN 76 ASN 77 SER 78 GLY 80 VAL 81 LEU 82 GLY 83  
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ALA 73 LEU 75 SER 78 ILE 79 GLY 80 VAL 81 LEU 82 GLY 83  
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ALA 73 LEU 75 ASN 77 SER 78 GLY 80 VAL 81 LEU 82 GLY 83  
ALA 73 LEU 75 ASN 77 SER 78 ILE 79 VAL 81 LEU 82 GLY 83  
ALA 73 LEU 75 ASN 77 SER 78 ILE 79 GLY 80 LEU 82 GLY 83  
ALA 73 LEU 75 ASN 77 SER 78 ILE 79 GLY 80 VAL 81 GLY 83  
ALA 73 LEU 75 ASN 77 SER 78 ILE 79 GLY 80 VAL 81 LEU 82  
ALA 73 LEU 75 ASN 76 ILE 79 GLY 80 VAL 81 LEU 82 GLY 83  
ALA 73 LEU 75 ASN 76 SER 78 GLY 80 VAL 81 LEU 82 GLY 83  
ALA 73 LEU 75 ASN 76 SER 78 ILE 79 VAL 81 LEU 82 GLY 83  
ALA 73 LEU 75 ASN 76 SER 78 ILE 79 GLY 80 LEU 82 GLY 83

ALA 73 LEU 75 ASN 76 SER 78 ILE 79 GLY 80 VAL 81 GLY 83  
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ALA 73 LEU 75 ASN 76 ASN 77 GLY 80 VAL 81 LEU 82 GLY 83  
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ALA 73 LEU 75 ASN 76 ASN 77 SER 78 ILE 79 GLY 80 VAL 81  
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ALA 73 ALA 74 ASN 77 ILE 79 GLY 80 VAL 81 LEU 82 GLY 83  
ALA 73 ALA 74 ASN 77 SER 78 GLY 80 VAL 81 LEU 82 GLY 83  
ALA 73 ALA 74 ASN 77 SER 78 ILE 79 VAL 81 LEU 82 GLY 83  
ALA 73 ALA 74 ASN 77 SER 78 ILE 79 GLY 80 LEU 82 GLY 83  
ALA 73 ALA 74 ASN 77 SER 78 ILE 79 GLY 80 VAL 81 GLY 83  
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ALA 73 ALA 74 ASN 76 ASN 77 SER 78 GLY 80 LEU 82 GLY 83  
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ALA 73 ALA 74 LEU 75 ASN 77 SER 78 ILE 79 GLY 80 VAL 81  
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ALA 73 ALA 74 LEU 75 ASN 76 ILE 79 GLY 80 VAL 81 GLY 83  
ALA 73 ALA 74 LEU 75 ASN 76 ILE 79 GLY 80 VAL 81 LEU 82  
ALA 73 ALA 74 LEU 75 ASN 76 SER 78 VAL 81 LEU 82 GLY 83

ALA 73 ALA 74 LEU 75 ASN 76 SER 78 GLY 80 LEU 82 GLY 83  
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 ALA 73 ALA 74 LEU 75 ASN 76 SER 78 ILE 79 GLY 80 LEU 82  
 ALA 73 ALA 74 LEU 75 ASN 76 SER 78 ILE 79 GLY 80 VAL 81  
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 ALA 73 ALA 74 LEU 75 ASN 76 ASN 77 GLY 80 LEU 82 GLY 83  
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 ALA 73 ALA 74 LEU 75 ASN 76 ASN 77 ILE 79 LEU 82 GLY 83  
 ALA 73 ALA 74 LEU 75 ASN 76 ASN 77 ILE 79 VAL 81 GLY 83  
 ALA 73 ALA 74 LEU 75 ASN 76 ASN 77 ILE 79 VAL 81 LEU 82  
 ALA 73 ALA 74 LEU 75 ASN 76 ASN 77 ILE 79 GLY 80 GLY 83  
 ALA 73 ALA 74 LEU 75 ASN 76 ASN 77 ILE 79 GLY 80 LEU 82  
 ALA 73 ALA 74 LEU 75 ASN 76 ASN 77 ILE 79 GLY 80 VAL 81  
 ALA 73 ALA 74 LEU 75 ASN 76 ASN 77 SER 78 LEU 82 GLY 83  
 ALA 73 ALA 74 LEU 75 ASN 76 ASN 77 SER 78 VAL 81 GLY 83  
 ALA 73 ALA 74 LEU 75 ASN 76 ASN 77 SER 78 VAL 81 LEU 82  
 ALA 73 ALA 74 LEU 75 ASN 76 ASN 77 SER 78 GLY 80 GLY 83  
 ALA 73 ALA 74 LEU 75 ASN 76 ASN 77 SER 78 GLY 80 LEU 82  
 ALA 73 ALA 74 LEU 75 ASN 76 ASN 77 SER 78 GLY 80 VAL 81  
 ALA 73 ALA 74 LEU 75 ASN 76 ASN 77 SER 78 ILE 79 GLY 83  
 ALA 73 ALA 74 LEU 75 ASN 76 ASN 77 SER 78 ILE 79 LEU 82  
 ALA 73 ALA 74 LEU 75 ASN 76 ASN 77 SER 78 ILE 79 VAL 81  
 ALA 73 ALA 74 LEU 75 ASN 76 ASN 77 SER 78 ILE 79 GLY 80

Table 10

## Nonuple Mutation Variants

LEU 75 ASN 76 ASN 77 SER 78 ILE 79 GLY 80 VAL 81 LEU 82 GLY 83  
 ALA 74 ASN 76 ASN 77 SER 78 ILE 79 GLY 80 VAL 81 LEU 82 GLY 83  
 ALA 74 LEU 75 ASN 77 SER 78 ILE 79 GLY 80 VAL 81 LEU 82 GLY 83  
 ALA 74 LEU 75 ASN 76 SER 78 ILE 79 GLY 80 VAL 81 LEU 82 GLY 83



ALA 74 LEU 75 ASN 76 ASN 77 ILE 79 GLY 80 VAL 81 LEU 82 GLY 83  
ALA 74 LEU 75 ASN 76 ASN 77 SER 78 GLY 80 VAL 81 LEU 82 GLY 83  
ALA 74 LEU 75 ASN 76 ASN 77 SER 78 ILE 79 VAL 81 LEU 82 GLY 83  
ALA 74 LEU 75 ASN 76 ASN 77 SER 78 ILE 79 GLY 80 LEU 82 GLY 83  
ALA 74 LEU 75 ASN 76 ASN 77 SER 78 ILE 79 GLY 80 VAL 81 GLY 83  
ALA 74 LEU 75 ASN 76 ASN 77 SER 78 ILE 79 GLY 80 VAL 81 LEU 82  
ALA 73 ASN 76 ASN 77 SER 78 ILE 79 GLY 80 VAL 81 LEU 82 GLY 83  
ALA 73 LEU 75 ASN 77 SER 78 ILE 79 GLY 80 VAL 81 LEU 82 GLY 83  
ALA 73 LEU 75 ASN 76 SER 78 ILE 79 GLY 80 VAL 81 LEU 82 GLY 83  
ALA 73 LEU 75 ASN 76 ASN 77 ILE 79 GLY 80 VAL 81 LEU 82 GLY 83  
ALA 73 LEU 75 ASN 76 ASN 77 SER 78 GLY 80 VAL 81 LEU 82 GLY 83  
ALA 73 LEU 75 ASN 76 ASN 77 SER 78 ILE 79 VAL 81 LEU 82 GLY 83  
ALA 73 LEU 75 ASN 76 ASN 77 SER 78 ILE 79 GLY 80 LEU 82 GLY 83  
ALA 73 LEU 75 ASN 76 ASN 77 SER 78 ILE 79 GLY 80 VAL 81 GLY 83  
ALA 73 LEU 75 ASN 76 ASN 77 SER 78 ILE 79 GLY 80 VAL 81 LEU 82  
ALA 73 ALA 74 ASN 77 SER 78 ILE 79 GLY 80 VAL 81 LEU 82 GLY 83  
ALA 73 ALA 74 ASN 76 SER 78 ILE 79 GLY 80 VAL 81 LEU 82 GLY 83  
ALA 73 ALA 74 ASN 76 ASN 77 ILE 79 GLY 80 VAL 81 LEU 82 GLY 83  
ALA 73 ALA 74 ASN 76 ASN 77 SER 78 GLY 80 VAL 81 LEU 82 GLY 83  
ALA 73 ALA 74 ASN 76 ASN 77 SER 78 ILE 79 VAL 81 LEU 82 GLY 83  
ALA 73 ALA 74 ASN 76 ASN 77 SER 78 ILE 79 GLY 80 LEU 82 GLY 83  
ALA 73 ALA 74 ASN 76 ASN 77 SER 78 ILE 79 GLY 80 VAL 81 GLY 83  
ALA 73 ALA 74 ASN 76 ASN 77 SER 78 ILE 79 GLY 80 VAL 81 LEU 82  
ALA 73 ALA 74 LEU 75 SER 78 ILE 79 GLY 80 VAL 81 LEU 82 GLY 83  
ALA 73 ALA 74 LEU 75 ASN 77 ILE 79 GLY 80 VAL 81 LEU 82 GLY 83  
ALA 73 ALA 74 LEU 75 ASN 77 SER 78 GLY 80 VAL 81 LEU 82 GLY 83  
ALA 73 ALA 74 LEU 75 ASN 77 SER 78 ILE 79 VAL 81 LEU 82 GLY 83  
ALA 73 ALA 74 LEU 75 ASN 77 SER 78 ILE 79 GLY 80 LEU 82 GLY 83  
ALA 73 ALA 74 LEU 75 ASN 77 SER 78 ILE 79 GLY 80 VAL 81 GLY 83  
ALA 73 ALA 74 LEU 75 ASN 77 SER 78 ILE 79 GLY 80 VAL 81 LEU 82  
ALA 73 ALA 74 LEU 75 ASN 76 ILE 79 GLY 80 VAL 81 LEU 82 GLY 83  
ALA 73 ALA 74 LEU 75 ASN 76 SER 78 GLY 80 VAL 81 LEU 82 GLY 83  
ALA 73 ALA 74 LEU 75 ASN 76 SER 78 ILE 79 VAL 81 LEU 82 GLY 83  
ALA 73 ALA 74 LEU 75 ASN 76 SER 78 ILE 79 GLY 80 LEU 82 GLY 83  
ALA 73 ALA 74 LEU 75 ASN 76 SER 78 ILE 79 GLY 80 VAL 81 GLY 83  
ALA 73 ALA 74 LEU 75 ASN 76 SER 78 ILE 79 GLY 80 VAL 81 LEU 82

ALA 73 ALA 74 LEU 75 ASN 76 ASN 77 GLY 80 VAL 81 LEU 82 GLY 83  
 ALA 73 ALA 74 LEU 75 ASN 76 ASN 77 ILE 79 VAL 81 LEU 82 GLY 83  
 ALA 73 ALA 74 LEU 75 ASN 76 ASN 77 ILE 79 GLY 80 LEU 82 GLY 83  
 ALA 73 ALA 74 LEU 75 ASN 76 ASN 77 ILE 79 GLY 80 VAL 81 GLY 83  
 ALA 73 ALA 74 LEU 75 ASN 76 ASN 77 ILE 79 GLY 80 VAL 81 LEU 82  
 ALA 73 ALA 74 LEU 75 ASN 76 ASN 77 SER 78 VAL 81 LEU 82 GLY 83  
 ALA 73 ALA 74 LEU 75 ASN 76 ASN 77 SER 78 GLY 80 LEU 82 GLY 83  
 ALA 73 ALA 74 LEU 75 ASN 76 ASN 77 SER 78 GLY 80 VAL 81 GLY 83  
 ALA 73 ALA 74 LEU 75 ASN 76 ASN 77 SER 78 GLY 80 VAL 81 LEU 82  
 ALA 73 ALA 74 LEU 75 ASN 76 ASN 77 SER 78 ILE 79 LEU 82 GLY 83  
 ALA 73 ALA 74 LEU 75 ASN 76 ASN 77 SER 78 ILE 79 VAL 81 GLY 83  
 ALA 73 ALA 74 LEU 75 ASN 76 ASN 77 SER 78 ILE 79 VAL 81 LEU 82  
 ALA 73 ALA 74 LEU 75 ASN 76 ASN 77 SER 78 ILE 79 GLY 80 GLY 83  
 ALA 73 ALA 74 LEU 75 ASN 76 ASN 77 SER 78 ILE 79 GLY 80 LEU 82  
 ALA 73 ALA 74 LEU 75 ASN 76 ASN 77 SER 78 ILE 79 GLY 80 VAL 81

Table 11 exemplifies non-limiting preferred double mutation variants having one or more deletions and one or more substitutions:

Table 11

$\Delta$ 73, Leu75Ile	$\Delta$ 73, Leu75Met
$\Delta$ 73, Asn76His	$\Delta$ 73, Ser78Asn
$\Delta$ 73, Ser78Thr	$\Delta$ 73, Ile79Leu
$\Delta$ 73, Ile79Met	$\Delta$ 73, Ile79Thr
$\Delta$ 73, Val81Thr	$\Delta$ 73, Leu82Phe
$\Delta$ 73, Leu82His	$\Delta$ 74, Leu82His
$\Delta$ 74, Leu82Phe	$\Delta$ 74, Val81Thr
$\Delta$ 74, Val81Thr	$\Delta$ 74, Ile79Thr
$\Delta$ 74, Ile79Met	$\Delta$ 74, Ile79Leu
$\Delta$ 74, Ser78Thr	$\Delta$ 74, Ser78Asn
$\Delta$ 74, Asn76His	$\Delta$ 74, Leu75Met
$\Delta$ 74, Leu75Ile	$\Delta$ 74, Ser78Asp
$\Delta$ 75, Asn76His	$\Delta$ 75, Ser78Asn

$\Delta$ 75, Ser78Thr	$\Delta$ 75, Ile79Leu
$\Delta$ 75, Ile79Met	$\Delta$ 75, Ile79Thr
$\Delta$ 75, Val81Thr	$\Delta$ 75, Leu82Phe
$\Delta$ 75, Leu82His	$\Delta$ 76, Leu75Ile
$\Delta$ 76, Leu75Met	$\Delta$ 76, Ser78Asn
$\Delta$ 76, Leu75Thr	$\Delta$ 76, Ile79Leu
$\Delta$ 76, Ile79Met	$\Delta$ 76, Ile79Thr
$\Delta$ 76, Val81Thr	$\Delta$ 76, Leu82Phe
$\Delta$ 76, Leu82His	$\Delta$ 77, Leu75Ile
$\Delta$ 77, Leu75Met	$\Delta$ 77, Asn76His
$\Delta$ 77, Ser78Asn	$\Delta$ 77, Ser78Thr
$\Delta$ 77, Ile79Leu	$\Delta$ 77, Ile79Met
$\Delta$ 77, Ile79Thr	$\Delta$ 77, Val81Thr
$\Delta$ 77, Leu82Phe	$\Delta$ 77, Leu82His
$\Delta$ 78, Leu82His	$\Delta$ 78, Leu75Ile
$\Delta$ 78, Leu75Met	$\Delta$ 78, Leu75Ile
$\Delta$ 78, Asn76His	$\Delta$ 78, Ile79Leu
$\Delta$ 78, Ile79Met	$\Delta$ 78, Ile79Thr
$\Delta$ 78, Val81Thr	$\Delta$ 78, Leu82Phe
$\Delta$ 79, Leu82His	$\Delta$ 79, Leu75Ile
$\Delta$ 79, Leu75Met	$\Delta$ 79, Asn76His
$\Delta$ 79, Ser78Asn	$\Delta$ 79, Ser78Thr
$\Delta$ 79, Val81Thr	$\Delta$ 79, Leu82Phe
$\Delta$ 80, Leu75Ile	$\Delta$ 80, Leu75Met
$\Delta$ 80, Asn76His	$\Delta$ 80, Ser78Asn
$\Delta$ 80, Ser78Thr	$\Delta$ 80, Ile79Leu
$\Delta$ 80, Ile79Met	$\Delta$ 80, Ile79Thr
$\Delta$ 80, Val81Thr	$\Delta$ 80, Leu82Phe
$\Delta$ 80, Leu75Ile	$\Delta$ 81, Leu75Ile

Δ 81, Leu75Met	Δ 81, Asn76His
Δ 81, Ser78Asn	Δ 81, Ser78Thr
Δ 81, Ile79Leu	Δ 81, Ile79Met
Δ 81, Ile79Thr	Δ 81, Leu82Phe
Δ 81, Leu82His	Δ 82, Leu75Ile
Δ 82, Leu75Met	Δ 82, Asn76His
Δ 82, Ser78Asn	Δ 82, Ser78Thr
Δ 82, Ile79Leu	Δ 82, Ile79Met
Δ 82, Ile79Thr	Δ 82, Val81Thr
Δ 83, Leu75Ile	Δ 83, Leu75Met
Δ 83, Asn76His	Δ 83, Ser78Asn
Δ 83, Ser78Thr	Δ 83, Ile79Leu
Δ 83, Ile79Met	Δ 83, Ile79Thr
Δ 83, Val81Thr	Δ 83, Leu82Phe

One or more additional substitution mutations ("stabilizing substitutions") outside the epitope region may additionally be made in order to, for example, restabilize the protease upon mutation of the epitope region or to enhance the proteolytic activity of the variant. Many such stabilizing mutations are well known in the art. Examples of such stabilizing mutations are disclosed in, for example, WO 95/10591, Baeck et al., published April 20, 1995; U.S. Pat. No. 4,914,031, Zukowski et al., issued April 3, 1990; U.S. Pat. No. 5,470,733, Bryan et al., issued November 28, 1995; U.S. Pat. No. 5,567,601, Bryan et al., issued October 22, 1996; WO 89/07642, U.S. Pat. No. 5,707,848, Bryan et al., issued January 13, 1998; Van Eekelen et al., published August 24, 1989; WO 87/04461, Stabinsky et al., published July 30, 1987; U.S. Pat. No. 4,760,025, Estell et al., issued July 26, 1988; WO 92/11348, Branner et al., published July 9, 1992; EP 0,405,901, Casteleijn et al., published January 2, 1991; WO 91/00345, Branner et al., published January 10, 1991; and WO 94/10020, Brode et al., published March 23, 1995.

Preferred stabilizing mutations include one or more of: I107V; K213R; Y217L; Y217K; N218S; G169A; M50F; Q19E; P5A; S9A; I31L; E156S; G169A; N212G; S188P; T254A; S3C + Q206C; and Q271E. The more preferred stabilizing mutations include one or more of P5A; S9A; I31L; E156S; G169A; N212G; S188P; T254A; S3C + Q206C; Q271E; Y217L; and Y217K. The most preferred stabilizing mutations include Y217L and Y217K.

A preferred variant for use in personal care compositions is  $\Delta$  75 - 83, Y217L.

#### Method of Making

The variants are prepared by mutating the nucleotide sequences that code for a wild-type serine protease, thereby resulting in variants having modified amino acid sequences. Such methods are well-known in the art; one such method is set forth below:

A phagemid (pSS-5) containing the wild-type subtilisin BPN' gene (Mitchison, C. and J.A. Wells, "Protein Engineering of Disulfide Bonds in Subtilisin BPN'", *Biochemistry*, Vol. 28, pp. 4807 - 4815 (1989) is transformed into *Escherichia coli dut-ung-* strain CJ236 and a single stranded uracil-containing DNA template is produced using the VCSM13 helper phage (Kunkel et al., "Rapid and Efficient Site-Specific Mutagenesis Without Phenotypic Selection", *Methods in Enzymology*, Vol 154, pp. 367 - 382 (1987), as modified by Yuckenberg et al., "Site-Directed *in vitro* Mutagenesis Using Uracil-Containing DNA and Phagemid Vectors", Directed Mutagenesis - A Practical Approach, McPherson, M. J. ed., pp. 27 - 48 (1991). Primer site-directed mutagenesis modified from the method of Zoller and Smith (Zoller, M. J., and M. Smith, "Oligonucleotide - Directed Mutagenesis Using M13 - Derived Vectors: An Efficient and General Procedure for the Production of Point Mutations in any Fragment of DNA", *Nucleic Acids Research*, Vol. 10, pp. 6487 - 6500 (1982) is used to produce all mutants (essentially as presented by Yuckenberg et al., *supra*).

Oligonucleotides are made using a 380B DNA synthesizer (Applied Biosystems Inc.). Mutagenesis reaction products are transformed into *Escherichia coli* strain MM294 (American Type Culture Collection *E. coli* 33625). All mutations are confirmed by DNA sequencing and the isolated DNA is transformed into the *Bacillus subtilis* expression strain PG632 (Saunders et al., "Optimization of the Signal-Sequence

Cleavage Site for Secretion from *Bacillus subtilis* of a 34-amino acid Fragment of Human Parathyroid Hormone", *Gene*, Vol. 102, pp. 277 - 282 (1991) and Yang et al., "Cloning of the Neutral Protease Gene of *Bacillus subtilis* and the Use of the Cloned Gene to Create an *in vitro* - Derived Deletion Mutation", *Journal of Bacteriology*, Vol. 160, pp. 15 - 21 (1984). Preliminary assessment of variant activity is determined by the ability of PG632 cells transformed with mutant plasmids to hydrolyze casein.

Fermentation is as follows. *Bacillus subtilis* cells (PG632) containing the variant of interest are grown to mid-log phase in one liter of LB broth containing 10 g/L glucose, and inoculated into a Biostat C fermentor (Braun Biotech, Inc., Allentown, PA) in a total volume of 9 liters. The fermentation medium contains yeast extract, casein hydrosylate, soluble - partially hydrolyzed starch (Maltrin M-250), antifoam, buffers, and trace minerals (see "Biology of Bacilli: Applications to Industry", Doi, R. H. and M. McGloughlin, eds. (1992)). The broth is kept at a constant pH of 7.5 during the fermentation run. Kanamycin (50 µg/mL) is added for antibiotic selection of the mutagenized plasmid. The cells are grown for 18 hours at 37 °C to an  $A_{600}$  of about 60 and the product harvested.

The fermentation broth is taken through the following steps to obtain pure variant. The broth is cleared of *Bacillus subtilis* cells by tangential flow against a 0.16 µm membrane. The cell-free broth is then concentrated by ultrafiltration with a 8000 molecular weight cut-off membrane. The pH is adjusted to 5.5 with concentrated MES buffer (2-(N-morpholino)ethanesulfonic acid). The variant is further purified by cation exchange chromatography with S-sepharose and elution with NaCl gradients. (see Scopes, R. K., "Protein Purification Principles and Practice", Springer-Verlag, New York (1984).

A pNA assay (DelMar et al., *Analytical Biochemistry*, Vol. 99, pp. 316 - 320 (1979)) is used to determine the active variant concentration for fractions collected during gradient elution. This assay measures the rate at which *p*-nitroaniline is released as the variant hydrolyzes the soluble synthetic substrate, succinyl-alanine-alanine-proline-phenylalanine-*p*-nitroaniline (sAAPF-pNA). The rate of production of yellow color from the hydrolysis reaction is measured at 410 nm on a spectrophotometer and is

proportional to the active enzyme concentration. In addition, absorbance measurements at 280 nm are used to determine the total protein concentration. The active enzyme/total-protein ratio gives the variant purity, and is used to identify fractions to be pooled for the stock solution.

To avoid autolysis of the variant during storage, an equal weight of propylene glycol is added to the pooled fractions obtained from the chromatography column. Upon completion of the purification procedure the purity of the stock variant solution is checked with SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) and the absolute enzyme concentration is determined *via* an active site titration method using trypsin inhibitor type II-T: turkey egg white (Sigma Chemical Company, St. Louis, MO).

In preparation for use, the enzyme stock solution is eluted through a Sephadex-G25 (Pharmacia, Piscataway, New Jersey) size exclusion column to remove the propylene glycol and exchange the buffer. The MES buffer in the enzyme stock solution is exchanged for 0.1 M tris buffer (tris(hydroxymethyl-aminomethane) containing 0.01M  $\text{CaCl}_2$  and pH adjusted to 8.6 with HCl. All experiments are carried out at pH 8.6 in tris buffer thermostated at 25 °C.

#### Analytical Methods

The present variants may be tested for enzymatic activity and immune and / or allergenic response using the following methods, both of which are known to one skilled in the art. Alternatively, other methods well-known in the art may be used.

#### Variant Activity

The protease activity of a variant of the present invention may be assayed by methods which are well-known in the art. Two such methods are set forth herein below:

##### Skin Flake Activity Method

Using Scotch® #3750G tape, human skin flakes are stripped from the legs of a subject repeatedly until the tape is substantially opaque with flakes. The tape is then cut into 1 inch by 1 inch squares and set aside. In a 10 mm by 35 mm petri dish, 2 mL of 0.75 mg / mL of a control enzyme (for example, subtilisin BPN') or the variant to be

tested is added in 0.01 M  $\text{KH}_2\text{PO}_4$  pH 5.5 buffer. To this solution 1 mL of 2.5% sodium laurate pH 8.6 solution is added. The solution is gently mixed on a platform shaker. The previously prepared tape square is soaked in the solution (flake side up) for ten minutes continuing gentle mixing. The tape square is then rinsed gently in tap water for fifteen seconds. Stevenel Blue Stain (3 mL, commercially available from Sigma Chemical Co., St. Louis, MO) is pipetted into a clean petri dish. The rinsed tape square is placed into the stain for three minutes (flake side up) with gentle mixing. The tape square is removed from the stain and rinsed consecutively in two beakers of 300 mL distilled water, for fifteen seconds per rinse. The tape square is allowed to air-dry. The color intensity between the tape square obtained from the control enzyme and the tape square obtained from the variant is compared visually or by using a chromameter. Relative to the control enzyme tape square, a variant tape square showing less color intensity is indicative of a variant having higher activity.

#### Dyed Collagen Activity Method

Combine 50 mL of 0.1 M tris buffer (tris-hydroxymethyl-aminomethane) containing 0.01 M  $\text{CaCl}_2$  to give pH 8.6, and 0.5 g azocoll (azo dye impregnated collagen, commercially available from Sigma Chemical Co., St. Louis, MO). Incubate this mixture at 25 °C while gently mixing with a platform shaker. Filter 2 mL of the mixture through a 0.2 micron syringe filter and read absorbance of the mixture at 520 nm to zero a spectrophotometer. Add 1 ppm of a control enzyme (for example, subtilisin BPN') or the variant to be tested to the remaining 48 mL of tris / azocoll mixture. Filter 2 mL of the control / variant containing solution through a 0.2 micron syringe filter every two minutes for a total of ten minutes. For each filtered sample, read the absorbance immediately at 520 nm. Plot the results against time. The slopes of the control and the test conjugate are indicative of relative activities of the samples. A higher slope is indicative of a higher activity. The test variant activity (slope) may be expressed as a percent of the control activity (slope).

#### T-Cell Proliferation Assay

The immune and / or allergenic potential of the variants of the present invention may be determined using a T-cell proliferation assay such as the assay presented



hereinbelow. This assay is a variation of the assay disclosed in Bungy Poor Fard et al., "T Cell Epitopes of the Major Fraction of Rye Grass *Lolium perenne* (*Lol p I*) Defined Using Overlapping Peptides in vitro and in vivo", *Clinical Experimental Immunology*, Vol. 94, pp. 111 - 116 (1993).

The blood of subjects allergic to subtilisin BPN' (prick test positive) and control subjects (prick test negative) are used in this assay. Blood (~ 60 mL) from each subject is collected and mononuclear cells are harvested using ficoll-hypaque (which may be obtained from Pharmacia, Piscataway, New Jersey). The cells are washed twice in RPMI 1640 (which may be obtained from Gibco, Grand Island, New York) and then resuspended in complete medium RPMI supplemented with 10% human AB-serum, 2 mM L-glutamine, and 25 µg / mL gentamicin (which may be obtained from Gibco). Cells are cultured at a concentration of  $2 \times 10^5$  cells / well in 0.2 mL of complete medium in U-bottomed 96-well microtiter plates. The potential antigen to be tested (either inactivated BPN' as positive control or a variant of the present invention) is added at a final concentration up to about 40 µg / mL. Cultures are incubated at 37 °C in 5% CO<sub>2</sub>. After five days, 1 µCi / well of methyl-<sup>3</sup>H-thymidine is added and 18 hours later the cells are harvested. <sup>3</sup>H-thymidine incorporation by the cell is assessed as a measure of T-cell proliferation by liquid scintillation counting.

#### Compositions of the Present Invention

The variants may be utilized in cleaning compositions including, but not limited to, laundry compositions, hard surface cleansing compositions, light duty cleaning compositions including dish cleansing compositions, and automatic dishwasher detergent compositions.

The cleaning compositions herein comprise an effective amount of one or more variants of the present invention and a cleaning composition carrier.

As used herein, "effective amount of variant", or the like, refers to the quantity of variant necessary to achieve the proteolytic activity necessary in the specific cleaning composition. Such effective amounts are readily ascertained by one of ordinary skill in the art and is based on many factors, such as the particular variant used, the cleaning application, the specific composition of the cleaning composition, and whether a liquid

or dry (e.g., granular, bar) composition is desired, and the like. Preferably, the cleaning compositions comprise from about 0.0001% to about 10%, more preferably from about 0.001% to about 1%, and most preferably from about 0.01% to about 0.1% of one or more variants of the present invention. Several examples of various cleaning compositions wherein the variants may be employed are discussed in further detail below.

In addition to the present variants, the present cleaning compositions further comprise a cleaning composition carrier comprising one or more cleaning composition materials compatible with the variant. The term "cleaning composition material", as used herein, means any material selected for the particular type of cleaning composition desired and the form of the product (e.g., liquid, granule, bar, spray, stick, paste, gel), which materials are also compatible with the variant used in the composition. The specific selection of cleaning composition materials is readily made by considering the material to be cleaned, the desired form of the composition for the cleaning condition during use. The term "compatible", as used herein, means the cleaning composition materials do not reduce the proteolytic activity of the variant to such an extent that the variant is not effective as desired during normal use situations. Specific cleaning composition materials are exemplified in detail hereinafter.

The variants of the present invention may be used in a variety of detergent compositions where high sudsing and good cleansing activity is desired. Thus, the variants can be used with various conventional ingredients to provide fully-formulated hard-surface cleaners, dishwashing compositions, fabric laundering compositions, and the like. Such compositions can be in the form of liquids, granules, bars, and the like. Such compositions can be formulated as "concentrated" detergents which contain as much as from about 30% to about 60% by weight of surfactants.

The cleaning compositions herein may optionally, and preferably, contain various surfactants (e.g., anionic, nonionic, or zwitterionic surfactants). Such surfactants are typically present at levels of from about 5% to about 35% of the compositions.

Nonlimiting examples of surfactants useful herein include the conventional C<sub>11</sub>-C<sub>18</sub> alkyl benzene sulfonates and primary and random alkyl sulfates, the C<sub>10</sub>-C<sub>18</sub>

secondary (2,3) alkyl sulfates of the formulas  $\text{CH}_3(\text{CH}_2)_x(\text{CHOSO}_3^- \text{M}^+)\text{CH}_3$  and  $\text{CH}_3(\text{CH}_2)_y(\text{CHOSO}_3^- \text{M}^+)\text{CH}_2\text{CH}_3$  wherein  $x$  and  $(y+1)$  are integers of at least about 7, preferably at least about 9, and  $\text{M}$  is a water-solubilizing cation, especially sodium, the  $\text{C}_{10}$ - $\text{C}_{18}$  alkyl alkoxy sulfates (especially EO 1-5 ethoxy sulfates),  $\text{C}_{10}$ - $\text{C}_{18}$  alkyl alkoxy carboxylates (especially the EO 1-5 ethoxycarboxylates), the  $\text{C}_{10}$ - $\text{C}_{18}$  alkyl polyglycosides, and their corresponding sulfated polyglycosides,  $\text{C}_{12}$ - $\text{C}_{18}$   $\alpha$ -sulfonated fatty acid esters,  $\text{C}_{12}$ - $\text{C}_{18}$  alkyl and alkyl phenol alkoxyates (especially ethoxyates and mixed ethoxy/propoxy),  $\text{C}_{12}$ - $\text{C}_{18}$  betaines and sulfobetaines ("sultaines"),  $\text{C}_{10}$ - $\text{C}_{18}$  amine oxides, and the like. The alkyl alkoxy sulfates (AES) and alkyl alkoxy carboxylates (AEC) are preferred herein. The use of such surfactants in combination with the amine oxide and / or betaine or sultaine surfactants is also preferred, depending on the desires of the formulator. Other conventional useful surfactants are listed in standard texts. Particularly useful surfactants include the  $\text{C}_{10}$ - $\text{C}_{18}$  N-methyl glucamides disclosed in U.S. Pat. No. 5, 194,639, Connor et al., issued March 16, 1993.

A wide variety of other ingredients useful in detergent cleaning compositions can be included in the compositions herein including, for example, other active ingredients, carriers, hydrotropes, processing aids, dyes or pigments, and solvents for liquid formulations. If an additional increment of sudsing is desired, suds boosters such as the  $\text{C}_{10}$ - $\text{C}_{16}$  alkalamides can be incorporated into the compositions, typically at about 1% to about 10% levels. The  $\text{C}_{10}$ - $\text{C}_{14}$  monoethanol and diethanol amides illustrate a typical class of such suds boosters. Use of such suds boosters with high sudsing adjunct surfactants such as the amine oxides, betaines and sultaines noted above is also advantageous. If desired, soluble magnesium salts such as  $\text{MgCl}_2$ ,  $\text{MgSO}_4$ , and the like, can be added at levels of, typically, from about 0.1% to about 2%, to provide additional sudsing.

The liquid detergent compositions herein may contain water and other solvents as carriers. Low molecular weight primary or secondary alcohols exemplified by methanol, ethanol, propanol, and *iso*-propanol are suitable. Monohydric alcohols are preferred for solubilizing surfactants, but polyols such as those containing from about 2 to about 6 carbon atoms and from about 2 to about 6 hydroxy groups (*e.g.*, 1,3-

propanediol, ethylene glycol, glycerine, and 1,2-propanediol) can also be used. The compositions may contain from about 5% to about 90%, typically from about 10% to about 50% of such carriers.

The detergent compositions herein will preferably be formulated such that during use in aqueous cleaning operations, the wash water will have a pH between about 6.8 and about 11. Finished products are typically formulated at this range. Techniques for controlling pH at recommended usage levels include the use of, for example, buffers, alkalis, and acids. Such techniques are well known to those skilled in the art.

When formulating the hard surface cleaning compositions and fabric cleaning compositions of the present invention, the formulator may wish to employ various builders at levels from about 5% to about 50% by weight. Typical builders include the 1-10 micron zeolites, polycarboxylates such as citrate and oxydisuccinates, layered silicates, phosphates, and the like. Other conventional builders are listed in standard formularies.

Likewise, the formulator may wish to employ various additional enzymes, such as cellulases, lipases, amylases and proteases in such compositions, typically at levels of from about 0.001% to about 1% by weight. Various deterative and fabric care enzymes are well-known in the laundry detergent art.

Various bleaching compounds, such as the percarbonates, perborates and the like, can be used in such compositions, typically at levels from about 1% to about 15% by weight. If desired, such compositions can also contain bleach activators such as tetraacetyl ethylenediamine, nonanoyloxybenzene sulfonate, and the like, which are also known in the art. Usage levels typically range from about 1% to about 10% by weight.

Soil release agents, especially of the anionic oligoester type, chelating agents, especially the aminophosphonates and ethylenediaminedisuccinates, clay soil removal agents, especially ethoxylated tetraethylene pentamine, dispersing agents, especially polyacrylates and polyasparatates, brighteners, especially anionic brighteners, suds suppressors, especially silicones and secondary alcohols, fabric softeners, especially smectite clays, and the like can all be used in such compositions at levels ranging from

about 1% to about 35% by weight. Standard formularies and published patents contain multiple, detailed descriptions of such conventional materials.

Enzyme stabilizers may also be used in the cleaning compositions. Such enzyme stabilizers include propylene glycol (preferably from about 1% to about 10%), sodium formate (preferably from about 0.1% to about 1%) and calcium formate (preferably from about 0.1% to about 1%).

The present variants are useful in hard surface cleaning compositions. As used herein "hard surface cleaning composition" refers to liquid and granular detergent compositions for cleaning hard surfaces such as floors, walls, bathroom tile, and the like. Hard surface cleaning compositions of the present invention comprise an effective amount of one or more variants of the present invention, preferably from about 0.001% to about 10%, more preferably from about 0.01% to about 5%, more preferably still from about 0.05% to about 1% by weight of variant of the composition. In addition to comprising one or more of the variants, such hard surface cleaning compositions typically comprise a surfactant and a water-soluble sequestering builder. In certain specialized products such as spray window cleaners, however, the surfactants are sometimes not used since they may produce a filmy and / or streaky residue on the glass surface.

The surfactant component, when present, may comprise as little as 0.1% of the compositions herein, but typically the compositions will contain from about 0.25% to about 10%, more preferably from about 1% to about 5% of surfactant.

Typically the compositions will contain from about 0.5% to about 50% of a detergency builder, preferably from about 1% to about 10%.

Preferably the pH should be in the range of from about 7 to about 12. Conventional pH adjustment agents such as sodium hydroxide, sodium carbonate or hydrochloric acid can be used if adjustment is necessary.

Solvents may be included in the compositions. Useful solvents include, but are not limited to, glycol ethers such as diethyleneglycol monohexyl ether, diethyleneglycol monobutyl ether, ethyleneglycol monobutyl ether, ethyleneglycol monohexyl ether, propyleneglycol monobutyl ether, dipropyleneglycol monobutyl ether, and diols such as

2,2,4-trimethyl-1,3-pentanediol and 2-ethyl-1,3-hexanediol. When used, such solvents are typically present at levels of from about 0.5% to about 15%, more preferably from about 3% to about 11%.

Additionally, highly volatile solvents such as *iso*-propanol or ethanol can be used in the present compositions to facilitate faster evaporation of the composition from surfaces when the surface is not rinsed after "full strength" application of the composition to the surface. When used, volatile solvents are typically present at levels of from about 2% to about 12% in the compositions.

Hard surface cleaning compositions of the present invention are illustrated by the following examples.

#### Examples 1 - 6

##### Liquid Hard Surface Cleaning Compositions

	Ex. 1	Ex. 2	Ex. 3	Ex. 4	Ex. 5	Ex. 6
$\Delta$ 75 - 82	0.05 %	0.50 %	0.02 %	0.03 %	0.30 %	0.05 %
EDTA	-	-	2.90 %	2.90 %	-	-
Sodium Citrate	-	-	-	-	2.90 %	2.90 %
NaC <sub>12</sub> Alkyl-benzene sulfonate	1.95 %	-	1.95 %	-	1.95 %	-
NaC <sub>12</sub> Alkylsulfate	-	2.20 %	-	2.20 %	-	2.20 %
NaC <sub>12</sub> (ethoxy) sulfate	-	2.20 %	-	2.20 %	-	2.20 %
C <sub>12</sub> Dimethylamine oxide	-	0.50 %	-	0.50 %	-	0.50 %
Sodium cumene sulfonate	1.30 %	-	1.30 %	-	1.30 %	-
Hexyl Carbitol	6.30 %	6.30 %	6.30 %	6.30 %	6.30 %	6.30 %
Water	90.4 %	88.3 %	87.53 %	85.87 %	87.25 %	85.85 %

All formulas are adjusted to pH 7.

In Examples 1 - 6, the variants recited in Tables 2 - 10, and the preferred variants cited herein, among others, are substituted for  $\Delta$  75 - 82, with substantially similar results.

In another embodiment of the present invention, dishwashing compositions comprise one or more variants of the present invention. As used herein, "dishwashing composition" refers to all forms of compositions for cleaning dishes including, but not limited to, granular and liquid forms. Dishwashing compositions of the present invention are illustrated by the following examples.

#### Examples 7 - 10

##### Liquid Dish Detergent

	Ex. 7	Ex. 8	Ex. 9	Ex. 10
$\Delta$ 79	0.05 %	0.50 %	0.02 %	0.40 %
C <sub>12</sub> - C <sub>14</sub> N-methyl glucamide	0.90 %	0.90 %	0.90 %	0.90 %
C <sub>12</sub> ethoxy (1) sulfate	12.0 %	12.0 %	12.0 %	12.0 %
2-Methyl undecanoic acid	4.50 %	4.50 %	4.50 %	4.50 %
C <sub>12</sub> ethoxy (2) carboxylate	4.50 %	4.50 %	4.50 %	4.50 %
C <sub>12</sub> alcohol ethoxylate (4)	3.00 %	3.00 %	3.00 %	3.00 %
C <sub>12</sub> amine oxide	3.00 %	3.00 %	3.00 %	3.00 %
Sodium cumene sulfonate	2.00 %	2.00 %	2.00 %	2.00 %
Ethanol	4.00 %	4.00 %	4.00 %	4.00 %
Mg <sup>2+</sup> (as MgCl <sub>2</sub> )	0.20 %	0.20 %	0.20 %	0.20 %
Ca <sup>2+</sup> (as CaCl <sub>2</sub> )	0.40 %	0.40 %	0.40 %	0.40 %
Water	65.45 %	65 %	65.48 %	65.1 %

All formulas are adjusted to pH 7.

In Examples 7 - 10, the variants recited in Tables 2 - 10, and the preferred variants cited herein, among others, are substituted for  $\Delta$  79, with substantially similar results.

Liquid fabric cleaning compositions of the present invention are illustrated by the following examples.

Examples 11 - 13

## Liquid Fabric Cleaning Compositions

	Ex. 11	Ex. 12	Ex. 13
$\Delta$ 70, 75 - 82	0.05 %	0.03 %	0.30 %
Sodium C <sub>12</sub> - C <sub>14</sub> alkyl sulfate	20.0 %	20.0 %	20.0 %
2-Butyl octanoic acid	5.0 %	5.0 %	5.0 %
Sodium citrate	1.0 %	1.0 %	1.0 %
C <sub>10</sub> alcohol ethoxylate (3)	13.0 %	13.0 %	13.0 %
Monoethanolamine	2.50 %	2.50 %	2.50 %
Water/propylene glycol/ethanol (100:1:1)	58.45 %	58.47 %	58.20 %

In Examples 11 - 13, the variants recited in Tables 2 - 10, and the preferred variants cited herein, among others, are substituted for  $\Delta$  70, 75 - 82, with substantially similar results.

Personal Care Compositions

The present variants are particularly suited for use in personal care compositions such as, for example, leave-on and rinse-off hair conditioners, shampoos, leave-on and rinse-off acne compositions, facial milks and conditioners, shower gels, soaps, foaming and non-foaming facial cleansers, cosmetics, hand, facial, and body lotions and moisturizers, leave-on facial moisturizers, cosmetic and cleansing wipes, oral care compositions, and contact lens care compositions. The present personal care compositions comprise one or more variants of the present invention and a personal care carrier.

To illustrate, the present variants are suitable for inclusion in the compositions described in the following references: U.S. Pat. No. 5,641,479, Linares et al., issued June 24, 1997 (skin cleansers); U.S. Pat. No. 5,599,549, Wivell et al., issued February 4, 1997 (skin cleansers); U.S. Pat. No. 5,585,104, Ha et al., issued December 17, 1996 (skin cleansers); U.S. Pat. No. 5,540,852, Kefauver et al., issued July 30, 1996 (skin cleansers); U.S. Pat. No. 5,510,050, Dunbar et al., issued April 23, 1996 (skin cleansers);



U.S. Pat. No. 5,612,324, Guang Lin et al., issued March 18, 1997 (anti-acne preparations); U.S. Pat. No. 5,587,176, Warren et al., issued December 24, 1996 (anti-acne preparations); U.S. Pat. No. 5,549,888, Venkateswaran, issued August 27, 1996 (anti-acne preparations); U.S. Pat. No. 5,470,884, Corless et al., issued November 28, 1995 (anti-acne preparations); U.S. Pat. No. 5,650,384, Gordon et al., issued July 22, 1997 (shower gels); U.S. Pat. No. 5,607,678, Moore et al., issued March 4, 1997 (shower gels); U.S. Pat. No. 5,624,666, Coffindaffer et al., issued April 29, 1997 (hair conditioners and / or shampoos); U.S. Pat. No. 5,618,524, Bolich et al., issued April 8, 1997 (hair conditioners and / or shampoos); U.S. Pat. No. 5,612,301, Inman, issued March 18, 1997 (hair conditioners and / or shampoos); U.S. Pat. No. 5,573,709, Wells, issued November 12, 1996 (hair conditioners and / or shampoos); U.S. Pat. No. 5,482,703, Pings, issued January 9, 1996 (hair conditioners and / or shampoos); U.S. Pat. No. Re. 34,584, Grote et al., Reissued April 12, 1994 (hair conditioners and / or shampoos); U.S. Pat. No. 5,641,493, Date et al., issued June 24, 1997 (cosmetics); U.S. Pat. No. 5,605,894, Blank et al., issued February 25, 1997 (cosmetics); U.S. Pat. No. 5,585,090, Yoshioka et al., issued December 17, 1996 (cosmetics); U.S. Pat. No. 4,939,179, Cheney et al., issued July 3, 1990 (hand, face, and / or body lotions); U.S. Pat. No. 5,607,980, McAtee et al., issued March 4, 1997 (hand, face, and / or body lotions); U.S. Pat. No. 4,045,364, Richter et al., issued August 30, 1977 (cosmetic and cleansing wipes); European Patent Application, EP 0 619 074, Touchet et al., published October 12, 1994 (cosmetic and cleansing wipes); U.S. Pat. No. 4,975,217, Brown-Skrobot et al., issued December 4, 1990 (cosmetic and cleansing wipes); U.S. Pat. No. 5,096,700, Seibel, issued March 17, 1992 (oral cleaning compositions); U.S. Pat. No. 5,028,414, Sampathkumar, issued July 2, 1991 (oral cleaning compositions); U.S. Pat. No. 5,028,415, Benedict et al., issued July 2, 1991 (oral cleaning compositions); U.S. Pat. No. 5,028,415, Benedict et al., issued July 2, 1991 (oral cleaning compositions); U.S. Pat. No. 4,863,627, Davies et al., September 5, 1989 (contact lens cleaning solutions); U.S. Pat. No. Re. 32,672, Huth et al., reissued May 24, 1988 (contact lens cleaning solutions); and U.S. Pat. No. 4,609,493, Schafer, issued September 2, 1986 (contact lens cleaning solutions).

To further illustrate oral cleaning compositions of the present invention, a pharmaceutically-acceptable amount of one or more variants of the present invention are included in compositions useful for removing proteinaceous stains from teeth or dentures. As used herein, "oral cleaning compositions" refers to dentifrices, toothpastes, toothgels, toothpowders, mouthwashes, mouth sprays, mouth gels, chewing gums, lozenges, sachets, tablets, biogels, prophylaxis pastes, dental treatment solutions, and the like. Preferably, the oral cleaning compositions comprise from about 0.0001% to about 20% of one or more variants of the present invention, more preferably from about 0.001% to about 10%, more preferably still from about 0.01% to about 5%, by weight of the composition, and a pharmaceutically-acceptable carrier. As used herein, "pharmaceutically-acceptable" means that drugs, medicaments or inert ingredients which the term describes are suitable for use in contact with the tissues of humans and lower animals without undue toxicity, incompatibility, instability, irritation, allergic response, and the like, commensurate with a reasonable benefit / risk ratio.

Typically, the pharmaceutically-acceptable oral cleaning carrier components of the oral cleaning compositions of the oral cleaning compositions will generally comprise from about 50% to about 99.99%, preferably from about 65% to about 99.99%, more preferably from about 65% to about 99%, by weight of the composition.

The pharmaceutically-acceptable carrier components and optional components which may be included in the oral cleaning compositions of the present invention are well known to those skilled in the art. A wide variety of composition types, carrier components and optional components useful in the oral cleaning compositions are disclosed in the references cited hereinabove.

In another embodiment of the present invention, denture cleaning compositions for cleaning dentures outside of the oral cavity comprise one or more variants of the present invention. Such denture cleaning compositions comprise an effective amount of one or more of the variants, preferably from about 0.0001% to about 50% of one or more of the variants, more preferably from about 0.001% to about 35%, more preferably still from about 0.01% to about 20%, by weight of the composition, and a denture cleansing carrier. Various denture cleansing composition formats such as effervescent tablets and

the like are well known in the art (see, e.g., U.S. Pat. No. 5,055,305, Young), and are generally appropriate for incorporation of one or more of the variants for removing proteinaceous stains from dentures.

In another embodiment of the present invention, contact lens cleaning compositions comprise one or more variants of the present invention. Such contact lens cleaning compositions comprise an effective amount of one or more of the variants, preferably from about 0.01% to about 50% of one or more of the variants, more preferably from about 0.01% to about 20%, more preferably still from about 1% to about 5%, by weight of the composition, and a contact lens cleaning carrier. Various contact lens cleaning composition formats such as tablets, liquids and the like are well known in the art and are generally appropriate for incorporation of one or more variants of the present invention for removing proteinaceous stains from contact lenses.

The contact lens cleaning composition embodiment of the present invention is illustrated by Examples 14 - 17.

#### Examples 14 - 17

##### Contact Lens Cleaning Solution

	Ex. 14	Ex. 15	Ex. 16	Ex. 17
$\Delta$ 75 - 82	0.01 %	0.5 %	0.1 %	2.0 %
Glucose	50.0 %	50.0 %	50.0 %	50.0 %
Nonionic surfactant (polyoxyethylene - polyoxypropylene copolymer)	2.0 %	2.0 %	2.0 %	2.0 %
Anionic surfactant (polyoxyethylene - alkylphenylether sodium sulfricester)	1.0 %	1.0 %	1.0 %	1.0 %
Sodium Chloride	1.0 %	1.0 %	1.0 %	1.0 %
Borax	0.30 %	0.30 %	0.30 %	0.30 %
Water	45.69 %	45.20 %	45.60 %	43.70 %

In Examples 14 - 17, the variants recited in Tables 2 - 10, and the preferred variants cited herein, among others, are substituted for  $\Delta$  75 - 82 with substantially similar results.

Examples 18 - 21 illustrate the use of the present variants in bodywash products:

Examples 18 - 21

Bodywash Products

	Ex. 18	Ex. 19	Ex. 20	Ex. 21
Water	62.62 %	65.72 %	57.72 %	60.72 %
Disodium EDTA	0.2 %	0.2 %	0.2 %	0.2 %
Glycerine	3.0 %	3.0 %	3.0 %	3.0 %
Polyquaternium 10	0.4 %	0.4 %	0.4 %	0.4 %
Sodium laureth sulphate	12.0 %	12.0 %	12.0 %	12.0 %
Cocamide MEA	2.8 %	2.8 %	2.8 %	2.8 %
Sodium lauraphoacetate	6.0 %	6.0 %	6.0 %	6.0 %
Myristic Acid	1.6 %	1.6 %	1.6 %	1.6 %
Magnesium sulphate heptahydrate	0.3 %	0.3 %	0.3 %	0.3 %
Trihydroxystearin	0.5 %	0.5 %	0.5 %	0.5 %
PEG-6 caprylic / capric triglycerides	3.0 %	-	-	-
Sucrose polyesters of cottonate fatty acid	3.0 %	-	-	-
Sucrose polyesters of behenate fatty acid	3.0 %	-	4.0 %	-
Petrolatum	-	4.0 %	8.0 %	-
Mineral Oil	-	-	-	6.0 %
DMDM Hydantoin	0.08 %	0.08 %	0.08 %	0.08 %
$\Delta$ 75 - 83, Y217L	0.1 %	2.0 %	2.0 %	5.0 %
Citric Acid	1.40 %	1.40 %	1.40 %	1.40 %

In Examples 18 - 21, the variants recited in Tables 2 - 10, and the preferred variants cited herein, among others, are substituted for  $\Delta$  75 - 83, Y217L, with substantially similar results.

Examples 18 - 21 illustrate the use of the present variants in facewash products:

#### EXAMPLES 22 - 25

##### Facewash Products

	Ex. 22	Ex. 23	Ex. 24	Ex. 25
Water	66.52 %	65.17 %	68.47 %	68.72 %
Disodium EDTA	0.1 %	0.1 %	0.2 %	0.2 %
Citric Acid	-	-	1.4 %	1.4 %
Sodium Laureth-3 Sulfate	3.0 %	3.5 %	-	-
Sodium Laureth-4 Carboxylate	3.0 %	3.5 %	-	-
Laureth-12	1.0 %	1.2 %	-	-
Polyquaternium 10	-	-	0.4 %	0.4 %
Polyquaternium 25	0.3 %	0.3 %	-	-
Glycerine	3.0 %	3.0 %	3.0 %	3.0 %
Sodium Lauroamphoacetate	-	-	6.0 %	6.0 %
Lauric Acid	6.0 %	6.0 %	3.0 %	3.0 %
Myristic Acid	-	-	3.0 %	3.0 %
Magnesium sulphate heptahydrate	2.3 %	2.0 %	2.0 %	2.0 %
Triethanol amine	4.0 %	4.0 %	4.0 %	4.0 %
Trihydroxystearin	0.5 %	0.5 %	0.5 %	0.5 %
Sucrose polyesters of behenate fatty acid	2.0 %	2.0 %	-	-
Sucrose polyesters of cottonate fatty acid	3.0 %	2.0 %	-	-
PEG-6 caprylic / capric triglycerides	-	-	-	2.0 %

Petrolatum	-	-	4.0 %	-
Mineral Oil	-	-	-	2.0 %
Cocamidopropyl betaine	2.0 %	3.0 %	1.8 %	1.8 %
Lauryl dimethylamine oxide	1.0 %	1.2 %	1.2 %	1.2 %
Dex Panthenol	1.0 %	0.25 %	0.25 %	-
DMDM Hydantoin	0.08 %	0.08 %	0.08 %	0.08 %
$\Delta$ 73, I79M	1.0 %	2.0 %	0.5 %	0.5 %
Fragrance	0.2 %	0.2 %	0.2 %	0.2 %

In Examples 22 - 25, the variants recited in Tables 2 - 10, and the preferred variants cited herein, among others, are substituted for  $\Delta$  73, I79M, with substantially similar results.

Examples 26 - 27 illustrate the use of the present variants in leave-on skin moisturizing compositions:

#### EXAMPLES 26 - 27

##### Leave-on Skin Moisturizing Composition

	Ex. 26	Ex. 27
Glycerine	5.0 %	-
Stearic acid	3.0 %	-
C <sub>11-13</sub> Isoparaffin	2.0 %	-
Glycol stearate	1.5 %	-
Propylene glycol	-	3.0 %
Mineral oil	1.0 %	10.0 %
Sesame oil	-	7.0 %
Petrolatum	-	1.8 %
Triethanolamine	0.7 %	-
Cetyl acetate	0.65 %	-
Glyceryl stearate	0.48 %	2.0 %
TEA stearate	-	2.5 %

Cetyl alcohol	0.47 %	-
Lanolin alcohol	-	1.8 %
DEA - cetyl phosphate	0.25 %	-
Methylparaben	0.2 %	0.2 %
Propylparaben	0.12 %	0.1 %
Carbomer 934	0.11 %	-
Disodium EDTA	0.1 %	-
$\Delta$ 75 - 83	0.1 %	0.5 %
Water	84.32 %	71.1 %

In Examples 26 - 27, the variants recited in Tables 2 - 10, and the preferred variants cited herein, among others, are substituted for  $\Delta$  75 - 83 with substantially similar results.

Example 28 illustrates the use of the present variants in cleansing wipe compositions:

#### EXAMPLE 28

##### Cleansing Wipe Composition

Propylene Glycol	1.0 %
Ammonium lauryl sulfate	0.6 %
Succinic acid	4.0 %
Sodium succinate	3.2 %
Triclosan®	0.15 %
$\Delta$ 75 - 82, G70V	0.05 %
Water	91.0 %

The above composition is impregnated onto a woven absorbent sheet comprised of cellulose and / or polyester at about 250 %, by weight of the absorbent sheet.

In Example 28, the variants recited in Tables 2 - 10, and the preferred variants cited herein, among others, are substituted for  $\Delta$  75 - 82, G70V with substantially similar results.



What is claimed is:

1. A variant of a serine protease characterized by a modified amino acid sequence of a wild-type amino acid sequence, wherein the modified amino acid sequence comprises a deletion of one or more of positions 70 - 84 corresponding to subtilisin BPN', wherein the variant is not  $\Delta$  75 - 83 or  $\Delta$  71.
2. A variant according to Claim 1 wherein the serine protease is selected from the group consisting of subtilisin BPN', subtilisin Carlsberg, subtilisin DY, subtilisin 309, proteinase K, and thermitase.
3. A variant according to Claims 1 or 2 wherein the deletion is of one or more of positions 73 - 83 corresponding to subtilisin BPN'.
4. A variant according to any of the preceding claims wherein the deletion is of one or more of positions 70 and 75 - 82 corresponding to subtilisin BPN'.
5. A variant according to any of the preceding claims selected from the group consisting of:
  - (a)  $\Delta$  70, 75 - 82;
  - (b)  $\Delta$  75 - 82;
  - (c)  $\Delta$  70, 78, 79;
  - (d)  $\Delta$  70;
  - (e)  $\Delta$  75;
  - (f)  $\Delta$  76;
  - (g)  $\Delta$  78;
  - (h)  $\Delta$  79;
  - (i)  $\Delta$  81; and
  - (j)  $\Delta$  82.

6. A variant according to any of the preceding claims further comprising a substitution of one or more of positions 70 - 84 corresponding to subtilisin BPN'.
7. A variant according to any of the preceding claims further comprising one or more stabilizing mutations.
8. A mutant serine protease gene encoding a variant according to any of the preceding claims.
9. A personal care composition characterized by:
  - (a) a variant of a serine protease having a modified amino acid sequence of a wild-type amino acid sequence, wherein the modified amino acid sequence comprises a deletion of one or more of positions 70 - 84 corresponding to BPN'; and
  - (b) a personal care composition carrier.
10. A composition according to Claim 9 wherein the variant is further characterized by a substitution at one or more of positions 70 - 84 corresponding to subtilisin BPN'.

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

- (i) APPLICANT: RUBINGH, D.N. et al.
- (ii) TITLE OF INVENTION: SERINE PROTEASE VARIANTS HAVING DECREASED IMMUNOGENICITY
- (iii) NUMBER OF SEQUENCES: 1
- (iv) CORRESPONDENCE ADDRESS:
  - (A) ADDRESSEE: THE PROCTER & GAMBLE COMPANY
  - (B) STREET: 11810 EAST MIAMI RIVER ROAD
  - (C) CITY: ROSS
  - (D) STATE: OH
  - (E) COUNTRY: USA
  - (F) ZIP: 45061
- (v) COMPUTER READABLE FORM:
  - (A) MEDIUM TYPE: Floppy disk
  - (B) COMPUTER: IBM PC compatible
  - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
  - (D) SOFTWARE: PatentIn Release #1.0, Version #1.30
- (vi) CURRENT APPLICATION DATA:
  - (A) APPLICATION NUMBER:
  - (B) FILING DATE:
  - (C) CLASSIFICATION:
- (viii) ATTORNEY/AGENT INFORMATION:
  - (A) NAME: HERSKO, BART S.
  - (B) REGISTRATION NUMBER: 32,572
  - (C) ATTORNEY DOCKET NO.
- (ix) TELECOMMUNICATION INFORMATION:
  - (A) TELEPHONE: 513-627-0633
  - (B) TELEFAX: 513-627-0260

## (2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 275 amino acids
  - (B) TYPE: amino acid
  - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: protein
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Ala	Gln	Ser	Val	Pro	Tyr	Gly	Val	Ser	Gln	Ile	Lys	Ala	Pro	Ala	Leu
1				5				10						15	
His	Ser	Gln	Gly	Tyr	Thr	Gly	Ser	Asn	Val	Lys	Val	Ala	Val	Ile	Asp
			20					25					30		
Ser	Gly	Ile	Asp	Ser	Ser	His	Pro	Asp	Leu	Lys	Val	Ala	Gly	Gly	Ala
			35				40						45		

Ser Met Val Pro Ser Glu Thr Asn Pro Phe Gln Asp Asn Asn Ser His  
 50 55 60  
 Gly Thr His Val Ala Gly Thr Val Ala Ala Leu Asn Asn Ser Ile Gly  
 65 70 75 80  
 Val Leu Gly Val Ala Pro Ser Ala Ser Leu Tyr Ala Val Lys Val Leu  
 85 90 95  
 Gly Ala Asp Gly Ser Gly Gln Tyr Ser Trp Ile Ile Asn Gly Ile Glu  
 100 105 110  
 Trp Ala Ile Ala Asn Asn Met Asp Val Ile Asn Met Ser Leu Gly Gly  
 115 120 125  
 Pro Ser Gly Ser Ala Ala Leu Lys Ala Ala Val Asp Lys Ala Val Ala  
 130 135 140  
 Ser Gly Val Val Val Val Ala Ala Ala Gly Asn Glu Gly Thr Ser Gly  
 145 150 155 160  
 Ser Ser Ser Thr Val Gly Tyr Pro Gly Lys Tyr Pro Ser Val Ile Ala  
 165 170 175  
 Val Gly Ala Val Asp Ser Ser Asn Gln Arg Ala Ser Phe Ser Ser Val  
 180 185 190  
 Gly Pro Glu Leu Asp Val Met Ala Pro Gly Val Ser Ile Gln Ser Thr  
 195 200 205  
 Leu Pro Gly Asn Lys Tyr Gly Ala Tyr Asn Gly Thr Ser Met Ala Ser  
 210 215 220  
 Pro His Val Ala Gly Ala Ala Ala Leu Ile Leu Ser Lys His Pro Asn  
 225 230 235 240  
 Trp Thr Asn Thr Gln Val Arg Ser Ser Leu Glu Asn Thr Thr Thr Lys  
 245 250 255  
 Leu Gly Asp Ser Phe Tyr Tyr Gly Lys Gly Leu Ile Asn Val Gln Ala  
 260 265 270  
 Ala Ala Gln  
 275

# INTERNATIONAL SEARCH REPORT

International Application No

PCT/IB 99/00520

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/57 C12N9/54 A61K7/00

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	W0 92 10755 A (NOVONORDISK AS) 25 June 1992 (1992-06-25)	1-10
Y	the whole document especially claim 1-14	9,10
A	WALSH B AND HOWDEN M: "A method for detection of IgE binding sequences of allergens based on a modification of epitope mapping" JOURNAL OF IMMUNOLOGICAL METHODS., vol. 121, 1989, pages 275-289, XP002112551 ELSEVIER SCIENCE PUBLISHERS B.V.,AMSTERDAM., NL ISSN: 0022-1759 cited in the application the whole document	1-10



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

### \* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search

19 August 1999

Date of mailing of the international search report

01/09/1999

Name and mailing address of the ISA

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Van der Schaal, C

# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/IB 99/00520

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 88 08028 A (GENEX CORP) 20 October 1988 (1988-10-20)	1-4, 6-8
Y	claims 1-20 ---	9, 10
A	WO 96 09396 A (UNIV MARYLAND) 28 March 1996 (1996-03-28) -----	

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/IB 99/00520

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